GENETIC ANALYSIS OF MITOCHONDRIAL PROTEIN TARGETING IN *SACCHAROMYCES CEREVISIAE*

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DECLARATION

I hereby declare that this thesis has composed by myself and that all the work described herein is my own.

Elizabeth M Ellis

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ABSTRACT

Most proteins which are located in the mitochondria of eukaryotic cells are synthesized as precursors on cytoplasmic ribosomes, and are post-translationally translocated across one or both mitochondrial membranes. The process of mitochondrial protein targeting is known to involve specific sequences which are usually located at the amino terminus of the mitochondrial precursor polypeptide. These presequences are thought to interact with cytoplasmic or mitochondrial components of the targeting machinery.

The research presented in this thesis describes approaches to identify such targeting components. Intially, the precursor of the matrix-located β-subunit of the F_1 ATPase from the yeast *Saccharomyces cerevisiae* was expressed in *Escherichia coli* and *S. cerevisiae* in an attempt to generate sufficient precursor protein for biochemical studies to allow the interaction between the precursor and components of the targeting machinery to be examined.

In order to characterize a mitochondrial presequence, deletion mutations in the presequence of β-subunit were constructed and their effect on targeting was examined. The deletion of amino acids 2 to 35 resulted in a reduction in targeting both *in vitro* and *in vivo,* whereas the deletion of amino acids 2 to 15 or 16 to 35 resulted in partial defects in either targeting or processing. The targeting defect caused by one of these smaller deletions (2-15) resulted in an unique phentoype when the yeast strain with this mutant protein was grown on glycerol as sole carbon source.

In an attempt to identify components which interact with mitochondrial presequences, extragenic suppressor mutants were isolated which could suppress the growth defect of the 2-15 deletion. One mutation (*mts1*) was isolated as a temperature-sensitive mutation in a nuclear gene. The cloning and sequencing of the wild-type *MTS1* gene has revealed that it is a novel gene, and an open reading frame of 1250 nucleotides has been identified. The *MTS1* gene is proposed to encode a protein of 48 kDa. Similarities between the MTS1 protein and known RNA-binding proteins suggest that it is an RNA-binding protein, and its possible role in the targeting pathway is discussed.

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ABBREVIATIONS

ADP - adenosine diphosphate

ATP - adenosine triphosphate

BPTI - bovine pancreatic trypsin inhibitor

BSA - bovine serum albumen

CCCP - carbanylcyanide *m*-chlorophenylhydrazone

COXII - cytochrome oxidase subunit II

COXIV - cytochrome oxidase subunit IV

dH_2O - distilled water

DHFR - dihydrofolate reductase

DNA - deoxyribonucleic acid

DTT - dithreothreitol

EDTA - ethylenediaminetetra acetic acid

ER - endoplasmic reticulum

$F_1\beta$ - β-subunit of F_1 ATPase

kb - kilobase

kDa - kilodaltons

OTC - orntihine transcarbamylase

OD - optical density

PCB - polypeptide chain binding (proteins)

PEP - phosphoenol pyruvate

Pi - inorganic phosphate

RNA - ribonucleic acid

RP - reverse primer

SDS- sodium dodecyl sulphate

SOD - superoxide dismutase

SRP - signal recognition particle

SSU - small subunit of ribulose bisphophate decarboxylase

TCA - trichloroacetic acid

TEMED - *NNNN*-tetramethylethylene diamine.

CHAPTER ONE

INTRODUCTION

1.1 Mitochondrial Protein Targeting.

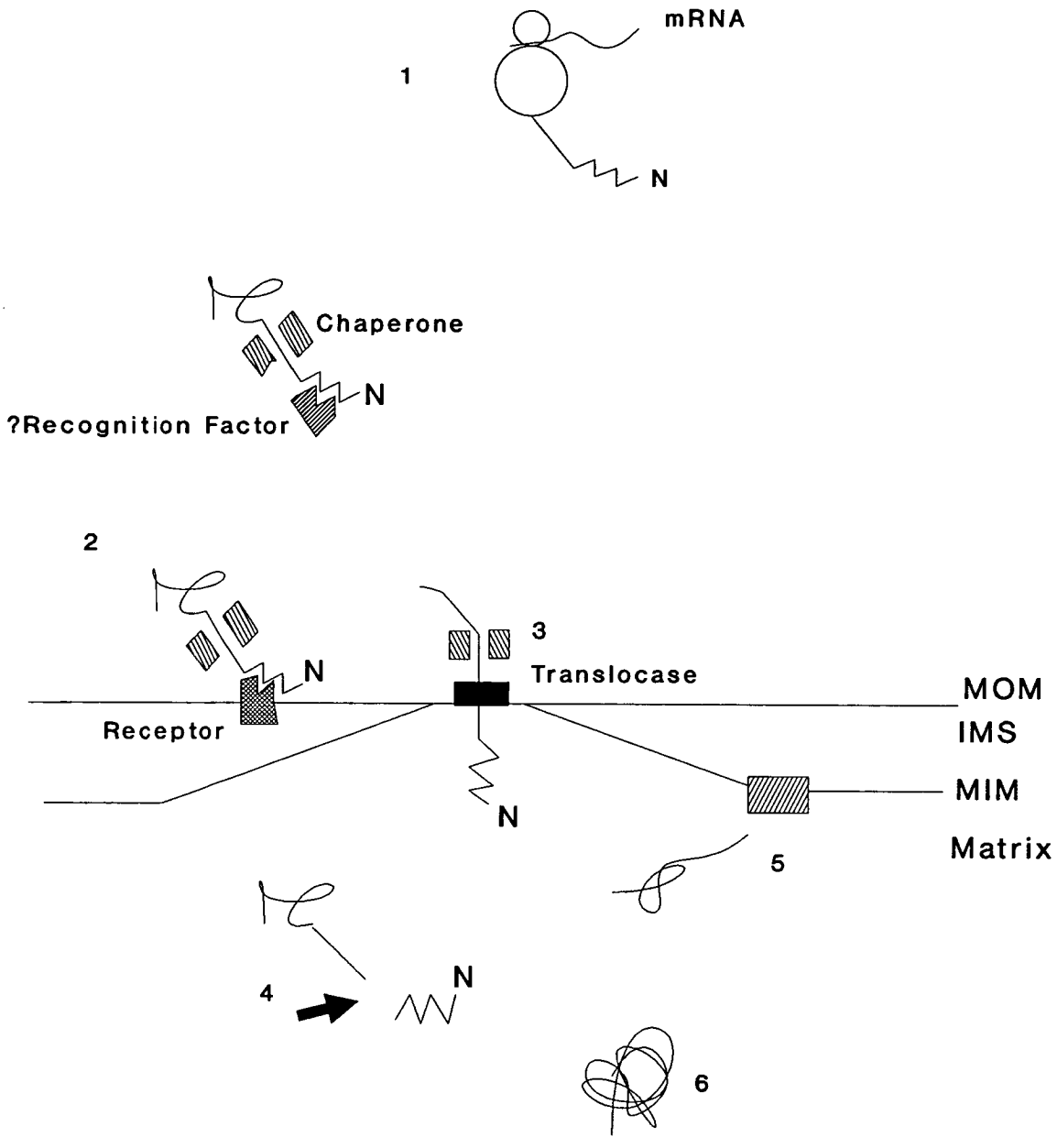
Eukaryotic cells are characterised by the compartmentalization of cellular functions. For example the mitochondrion is responsible for oxidative phosphorylation and several essential biosynthetic processes. The mitochondrion contains genetic information for only a very small number of proteins, and the machinery necessary for the transcription and translation of these genes (Tzagaloff, 1986). However, most proteins found in the mitochondria are encoded by genes located in the nucleus and are translated from messenger RNA (mRNA) on cytosolic ribosomes, usually as larger molecular weight 'precursors'. Several events must take place so that the precursor protein can assume its functional role. These can be broken down into distinct steps, which do not necessarily relate to separate physical events:

1. Synthesis of a precursor polypeptide;
2. Targeting to the mitochondrial outer membrane, and subsequent binding;
3. Energy-dependent translocation across one or both membranes;
4. Cleavage to the mature form;
5. Intramitochondrial sorting;
6. Assembly into functional complexes.

These steps are outlined in Figure 1.1. The process of mitochondrial protein targeting can therefore be defined as the mechanism by which the precursor polypeptide forms an interaction with the mitochondria which allows it to be subsequently translocated across the mitochondrial membrane(s) and assembled.

In order to discover the mechanism of this process, many studies have been carried out using various eukaryotic systems. Mitochondria from rat liver cells, *Saccharomyces cerevisiae* and *Neurospora crassa* have been

Figure 1.1 Pathway of Mitochondrial Protein Targeting



Mitochondrial precursors are synthesized on cytoplasmic ribosomes (1) and are recognized and targeted to the mitochondria, where they bind to receptors located in the mitochondrial outer membrane (MOM) (2). Cytoplasmic molecular chaperones are thought to confer import competence for the energy-dependent translocation step, and a membrane bound translocase is thought to be required (3). Precursors are cleaved by a matrix-located protease (4), and proteins destined for the intermembrane space (IMS) or inner membrane (MIM) are retargeted (5). Finally, proteins fold and assemble into functional complexes (6)

used with mitochondrial precursor polypetides originating from *S. cerevisiae, N. crassa*, rats and humans in *in vitro* systems. For *in vivo* studies, *S. cerevisiae*, and *N. crassa* are the most widely used organisms. The process of mitochondrial protein targeting appears to be identical in all systems studied, thus allowing direct extrapolation of results from one system to another. Therefore, examples used in this Chapter to describe the advances already made in understanding the process of mitochondrial protein targeting are taken from a variety of systems. However, as the yeast *Saccharomyces cerevisiae* is amenable to biochemical and genetic analysis, coupled with the fact that it can grow fermentatively without the need for mitochondrial respiration, it is an ideal organism for the study of mitochondrial biogenesis. Therefore, the experimental work described in this thesis relates to yeast as a model for this fundamental process.

It is thought that the process of mitochondrial protein targeting might be analogous to other targeting processes, such as eukaryotic protein secretion, bacterial protein export, chloroplast targeting and perhaps nuclear targeting. The requirements of each system for efficient localization are summarized in Table 1.1. Many studies have already made progress in solving the problem of how precursors from these similar systems are recognized, targeted to the correct membrane and translocated, and as there may be a mechanistic overlap with mitochondrial targeting, these aspects are also discussed in this Chapter.

1.2 SYNTHESIS OF PRECURSOR POLYPEPTIDES.

As most mitochondrial proteins are encoded by nuclear genes, their mRNA transcripts exit from the nucleus and like cytoplasmic proteins, are translated by cytoplasmic ribosomes. After synthesis, mitochondrial polypeptides are distinguished in some way from other proteins, and are specifically localized to the mitochondrion. The features which appear to identify a polypeptide that is destined for the mitochondria are discussed in this section.

Legend to Table 1.1

Key

1 Walter and Blobel, 1980.

2 Sollner *et al.,* 1989.

3 Ohba and Schatz, 1987.

4 Schmidt *et al.,* 1988.

5 Ito, 1984.

6 Meyer and Dobberstein, 1980.

7 Bernstein *et al.,* 1989

8 Pain *et al.,* 1988.

9 Deshaies *et al.,* 1988.

10 Crooke and Wickner, 1987.

11 Collier *et al.,* 1988.

12 Chirico *et al.,* 1988.

13 Gilmore and Blobel, 1985.

14 Jensen and Yaffe, 1988; Pollock *et al.,*1988.

15 Zwizinski and Wickner, 1980.

16 Evans *et al.,* 1986.

17 Robinson and Ellis, 1984.

18 Cheng *et al.,* 1989.

19 Bole *et al.,* 1986.

20 Ellis and van der Vies, 1988.

21 Sanz and Meyer, 1989.

22 Vestweber *et al.,* 1989.

Table 1.1 Comparison of mitochondrial protein targeting and other protein targeting processes.

STEP	REQUIREMENT	MITOCHONDRIA TARGETING	BACTERIA EXPORT	EUKARYOTIC SECRETION	CHLOROPLAST TARGETING
Precursor synthesis	Targeting sequence	Presequence	Leader sequence	Signal sequence	Transit peptide
Recognition	Cytosolic factors	?	?	SRP[1]	?
	Receptors	MOM19[2] ?ISP42[3]	?SecA[4] ?SecY[5]	SRP receptor[6] SP Receptor[7]	30 kDa SSU[8]
	ATP for binding	x	x	x	✓
Translocation	Unfolded Precursor	✓	✓	✓	✓
	Chaperones	Hsp70[9]	Trigger Factor[10] SecB[11]	Hsp70[12] SRP[21]	?
	ATP requirement	✓	✓	✓	?
	Contact Sites	✓	✓	-	✓
	$\Delta\psi$	✓	✓	x	x
	Translocase	?ISP42[22]	?SecY[5]	?Pore[13]	?
Processing	Peptidase	MPP, PEP[14]	Leader Peptidase[15]	Signal Peptidase[16]	Stromal peptidase[17]
Sorting & assembly	Chaperones	Hsp60[18]	?	BiP[19]	Rubisco binding protein[20]

1.2.1 The Signal Hypothesis in Protein Targeting.

In order to achieve the specificity of targeting to a particular biological membrane or organelle, a targeted protein contains information for its own localization. Evidence for the existence of such "signal sequences" within the protein was first obtained with protein secretion into the endoplasmic reticulum (ER) of eukaryotic cells. Initial biochemical studies showed that proteins destined to be secreted are synthesized on ER membrane-bound polysomes, and are translocated across the membrane before their synthesis is complete (Redman *et al.,* 1966). In the light of this phenomenon, coupled with the fact that the carboxy terminus of the protein could be removed without affecting localization (Redman and Sabatini, 1966), it was postulated that it is the amino terminal end of the protein which contains the information for the correct localization of secreted proteins. The demonstration of the existence of a precursor protein (Milstein *et al.,* 1972) and the amino acid sequencing of such a precursor (Schechter *et al.,* 1975) supported this hypothesis; and the development of an *in vitro* system for assaying transport further confirmed the theory that the amino terminus acts as a signal peptide (Blobel and Dobberstein, 1975). The theory has been extended to include the export of proteins in *Escherichia coli*, where an amino terminal leader sequence has been shown to be required for efficient export (Inouye and Beckwith, 1977; Randall and Hardy, 1989).

Subsequently, mitochondrial protein targeting has been shown to share similarities with these other targeting processes, in that mitochondrial proteins are synthesized as precursors and possess cleavable signal sequences known as presequences. The requirement for precursors was first observed with three subunits of the F_1 ATPase complex of yeast (Maccecchini *et al.*, 1979). Total yeast mRNA was translated using an *in vitro* rabbit reticulocyte lysate translation system, and the radioactive proteins produced were immunoprecipitated using antibodies to the mature form of each of the α, β and γ subunits. Analysis of these proteins by SDS polyacrylamide gel electrophoresis showed that each protein was larger than

the corresponding mature protein by between 2,000 and 6,000 Daltons. Incubation of the precursor protein with isolated yeast mitochondria resulted in its import into the mitochondria in the absence of protein synthesis, and its conversion to the mature size (Maccechini *et al.,* 1979). This conversion was later shown to be due to proteolytic cleavage within the mitochondria (McAda and Douglas, 1982). Incubation of mature protein with isolated mitochondria did not result in the import of the protein, thus confirming the role of the precursor in the import process. Most nuclear-encoded mitochondrial proteins were subsequently shown to be synthesised as larger precursors, with a few exceptions (Section 1.2.2).

1.2.2 Nature of Targeting Information on Mitochondrial Precursors.

Cloning and sequencing of more than twenty of the nuclear genes encoding mitochondrial proteins from several different organisms has revealed features which may be important for their targeting. It was noticed that the amino acid sequence deduced from the DNA sequence of most of the cloned genes was larger than the mature protein by between 20 and 80 amino acids, and that this extension was located at the amino terminus. This corresponds with the observed molecular weight difference of the precursor and mature forms, and confirmed the presence of a presequence.

Not all mitochondrial proteins appear to possess cleavable presequences (Muesch *et al.,* 1990). For example the 70kDa outer membrane protein (Hase *et al.,* 1984), porin (outer membrane) (Freitag, *et al.,* 1982), and isopropylmalate synthase (matrix) (Hampsey *et al.,* 1983) are not cleaved. It has been demonstrated that the targeting information for these proteins is carried in the extreme amino terminal region of the mature protein (Hase *et al.,* 1984), and it is thought that this region functions in the same way as a cleavable presequence. However, the mitochondrial protein cytochrome c does not possess an amino terminal extension and is thought to follow a different pathway from most other mitochondrial precursors (Stuart *et al.,* 1990).

The physiological importance of an amino terminal extension for the correct cellular localization of a protein has been demonstrated experimentally for mitochondrial protein targeting, and is similar to that found in a wide variety of biological systems, both eukaryotic and prokaryotic (reviewed in Silhavy *et al.,* 1983; Zimmerman and Meyer, 1986). Wild-type and fusion proteins have been used, together with deletion analysis, to define the precise region of the presequence which was reponsible for the localization of the precursor protein, both *in vivo* and *in vitro.* (reviewed by Hurt and van Loon, 1986). This aspect is discussed in detail in Section 4.1.

Recently regions other than the extreme amino terminus of mitochondrial precursors have been implicated in the targeting process. For example, the carboxy terminal two-thirds of ADP/ATP carrier were able to direct the protein to mitochondria with the same efficiency as wild type precursor (Pfanner *et al.,* 1987b; Smagula and Douglas, 1988). Using fusions, Pfanner *et al.*, (1987c) have shown that a hydrophobic region within the mature part of the *N. crassa* F_0ATPase subunit 9 increases the efficiency of binding to mitochondria. This suggests that the mature part of this protein may also play a part in the import process. It is not known whether these examples represent a more general phenomenon.

As it is the amino terminal presequence in most cases which appears to dictate correct localization, how does it exert its effect? There appear to be four requirements for the function of a presequence. Firstly it must promote binding of the precursor to the correct intracellular membrane, and therefore must contain some degree of specificity. Secondly, it must promote translocation across the mitochondrial membrane, but this second requirement may be a direct result of the initial binding. Thirdly, as most precursors are processed, it must promote processing at a specific cleavage site. Finally, it must contain information for intramitochondrial sorting where the final destination is not the matrix. Mutagenesis and deletions within presequences has revealed that these functions may reside in different portions of the presequence (Chu *et al.,* 1989; Pfanner *et al.*, 1987c; Hurt *et*

al., 1987). Examples of mitochondrial precursors exhibiting these features are illustrated in Table 1.2.

1.2.3 Features Specifying Initial Interactions.

From fusion studies, it is clear that the minimum region of a presequence for localization to the mitochondrial matrix can be as short as 9 amino acids in the case of δ-aminolevulinate synthase (Keng *et al.,* 1986). Therefore, the extreme amino terminus of mitochondrial precursors has been proposed to be entirely responsible for the initial interaction with the mitochondria, and translocation into the matrix. It has been proposed that the features of the presequence responsible for this initial localization lie either in the structure of the presequence or in the conformation of the entire precursor which may be altered by the presence of the presequence, either directly or in concert with other proteins.

1.2.3.1 Structure of Presequences

Although there is no consensus sequence for a presequence, comparison of the presequences of many mitochondrial precursor proteins revealed certain general features, particularly at the amino terminus (Watson, 1984; von Heijne, 1985; von Heijne *et al.,* 1989). Firstly, there is an abundance of positively charged lysine and arginine residues. Secondly there are no or few acidic residues. Thirdly, there are many of the hydroxylated amino acids serine and threonine. These give the amino terminus a net positive charge. The construction of α-helical wheel plots of many mitochondrial presequences did show similarities between them (von Heijne, 1986): one face of the helix is positively charged, and the geometrically opposite face is hydrophobic. Such an amphiphilic structure would perhaps be capable of being recognized by the mitochondrion (Roise *et al.,* 1986).

The importance of an amphiphilic helix in mitochondrial targeting has since been demonstrated. Several artificial presequences containing only the amino acids leucine, arginine and serine arranged in such a way as to give amphiphilic helices, were capable of directing yeast cytochrome oxidase

Table 1.2 Features of Mitochondrial Targeting Sequences.

DESTINATION	PRECURSOR	DOMAINS
Matrix	F_1 β-subunit	Matrix Targeting Cleavage _____ .?↓ ?↓ + + + -+ + + MVLPRLYTATSRAAFKAAKQSAPLLSTSWKCMASAA
Intermembrane Space	Cytochrome b_2	Matrix Targeting 1st Cleavage Retargeting Domain 2nd Cleavage _____ ↓ ---------- ↓ + + + + + + + . - +++ + -. MLKYPLKISKNCEAAILRASLTRLNTIRAYGSTVPLSFEQDSRKRTQSWTALRVGAILAATSSVAYLNWHNGQIDN
Inner Membrane	Cytochrome c oxidase subunit 4 (COXIV)	Matrix Targeting Cleavage _____ ↓ + + + + + + MLSLRQSIRFFKPATRLCSSRYLL
Outer membrane	70 kDa OMP	Matrix Targeting Anchor Domain _____ (Uncharged) ++ + + ---------- + ++ MKSFITRNKTAILATVAATAIGAYYYYNQLQQQQQRGKK

Key

___ - matrix targeting domains
\+ - positively charged amino acids
\- - negatively charged amino acids
---- - sorting domains
↓ - cleavage sites

subunit IV (COXIV) to yeast mitochondria *in vivo* and *in vitro* (Allison and Schatz, 1986). More recently Roise *et al.* (1988) have shown that amphiphilicity is essential for function, but that a helix is not necessarily required. As the amphiphilic nature of the presequence seems to be important for targeting, how is it recognized ? It has been proposed that the ability to form an amphiphilic helix may allow the precursor protein to interact with the lipid bilayer, such as the mitochondrial outer membrane. The incorporation of a mitochondrial signal peptide into phospholipid monolayers has been demonstrated (Tamm, 1986). Evidence for the interaction of a synthetic peptide corresponding to the presequence of yeast cytochrome c oxidase subunit IV (COXIV) with a membrane was shown by Roise *et al.* (1986). However, an interaction with the membrane alone could not account for the specificity or efficiency of import of mitochondrial precursors - for example, why does the precursor interact only with the mitochondrial membrane, and not with the endoplasmic reticulum or nuclear membrane? It is probable therefore that the presequence must interact with other proteins in the cell to achieve this specificity (Section 1.3), and the structure of the presequence or parts of the presequence may be important for this.

Mitochondrial targeting signals are functionally similar to other types of targeting signal in both prokaryotic and eukaryotic systems. However they do not appear to show extensive similarities in primary structure with leader sequences for bacterial export or signal sequences for eukaryotic secretion. These two types of targeting signals seem to possess three distinct regions: a positively charged amino terminal region; a central hydrophobic region; and a polar carboxy terminal region which contains the cleavage site. Despite these differences from mitochondrial presequences, the importance of secondary structure in the functioning of bacterial signal sequences in protein export has also been identified (Emr and Silhavy, 1983). Using genetic techniques, the presence of an α-helix in the hydrophobic central region has been correlated with the ability to be exported (Emr and Silhavy, 1983; Briggs

and Gierasch, 1984), thus suggesting a common theme in protein targeting sequence structure.

Chloroplast transit peptides are the most similar to mitochondrial presequences. Like mitochondrial presequences, they are rich in basic and hydroxylated amino acids and do not contain acidic amino acids (von Heijne *et al.,* 1989). However they differ in that they are not enriched for arginine or lysine, and have an abundance of serine residues. Regions of the transit peptide are not thought to form amphiphilic α-helices, but amphiphilic β-sheets, and it is possible that these regions may function in a similar way to mitochondrial localization signals.

1.2.3.2 Precursor Conformation

Several reports suggest that the precursors of certain mitochondrial proteins have a different conformation from the mature protein. For example. the precursor of subunit 9 of *N. crassa* ATP synthase (Viebrock *et al.,* 1982); apocytochrome c from yeast (Korb and Neupert, 1978); and malate dehydrogenase from mammalian cells (Chien and Freeman, 1986). It is possible that the precursor adopts this conformation due to the presence of the presequence, a theory first proposed by Wickner (1979) as part of the membrane trigger hypothesis.

How could a targeting sequence cause the precursor to adopt a different conformation ? It is possible that the folding of the precursor is dictated by the presence of the presequence purely on thermodynamic rules. However fusion proteins have been made by fusing in frame the amino terminal region of a mitochondrial precursor protein to a protein not normally found in the mitochondria (such as dihydrofolate reductase or β-galactosidase). These proteins are targeted to the mitochondria *in vitro*, and at the same time they retain their enzymatic activity, even with the presequence attached (Eilers and Schatz, 1986). This suggests that the presequence has little direct effect on the overall structure of these fusion proteins under the conditions tested. However, this may not be the case for natural mitochondrial precursors,

which may have a less rigid structure. Another means by which the presequence could influence the structure of the precursor is by allowing the precursor to interact with other proteins, which are only present in the cytoplasm to elicit a change in or to maintain a specific conformation (Section 1.4.1.1).

There are several explanations as to how the conformation of a precursor could have an effect on the protein localization process. Firstly, an altered conformation could expose the presequence such that it is able to interact with a component of the targeting machinery in the initial binding step (Section 1.3). Secondly, an unfolded structure may be required for translocation (Section 1.4.1). The role of precursor structure in the translocation step has been demonstrated by Eilers and Schatz (1986), who showed that a fusion protein of COXIV presequence and DHFR could not be imported into yeast mitochondria when methotrexate was bound to the DHFR moiety. It was suggested that this was because the fusion protein was unable to unfold, and could not be translocated in the folded state (Eilers and Schatz, 1986). Further evidence of the importance of the conformational structure of the mature part of the protein for translocation was shown by the different efficiency of targeting of different fusion proteins (van Steeg *et al.*, 1986). The presequence of superoxide dismutase (SOD) was unable to efficiently target yeast invertase to the mitochondrial matrix *in vivo* in yeast, and it was suggested that this was due to the stable post-translational conformation adopted by this normally co-translationally translocated protein.

By comparison, studies in *E. coli* have also emphasized the importance of precursor structure in the export of proteins. Using proteases, Randall and Hardy (1986) showed that export of maltose-binding protein was dependent on the lack of tertiary structure and that it was unable to be translocated in a stably-folded conformation. It was also shown that a mutation in the leader sequence of a bacterial protein which prevented export correlated with an increase in the folding of the nascent protein (Randall and Hardy, 1986).

1.2.4 Features Specifying Processing.

Most mitochondrial precursor polypeptides undergo a processing step which removes most if not all of the amino terminal targeting signal. A matrix-located metalloprotease has been identified which cleaves a variety of mitochondrial precursors (McAda and Douglas, 1982), but no consensus sequence for processing has been identified within these precursors. As the processing protease fails to cleave denatured precursors, it has been suggested that the enzyme recognizes a three-dimensional structure which mitochondrial presequences possess (Jensen and Yaffe, 1988). In contrast to the wide range of mutations which can be accomodated in amino terminal targeting domain without affecting function, relatively small changes in cleavage domains can prevent processing (Hurt *et al.,* 1987; Nguyen *et al.,* 1987).

Some precursors such as cytochrome b_2 and cytochrome c_1 are processed to an intermediate sized protein before being processed to the mature size which suggests that two cleavage sites exist (Gasser *et al.,* 1982b). The extreme amino-terminus is cleaved by the matrix protease and the second processing event is carried out by an enzyme located in the inter membrane space. The sequences which specify this second cleavage event have been identified in the case of cytochochrome b_2 and subunit II of cytochrome oxidase (COXII) as Asn-Glu and Asn-Asp respectively (Taylor, 1989; Pratje and Guiard, 1986).

1.2.5 Features Specifying Intramitochondrial Sorting.

After interacting with the mitochondrial outer membrane, precursor proteins are translocated across the mitochondrial membrane(s) to their correct submitochondrial location. The protein's final destination can be one of four locations: the outer membrane; the inter-membrane space; the inner membrane and the matrix (Figure 1.1). Therefore the precursor polypeptide must contain not only information for targeting to the mitochondria but also information for intramitochondrial sorting. It is thought that most precursor

polypeptides contain information for localization to the mitochondrial matrix at the extreme amino terminus, and that precursors whose destination is not the matrix possess additional signals which specify intramitochondrial sorting (Hurt and van Loon, 1986).

Mitochondrial outer membrane proteins appear to posses an anchor element after the matrix-targeting domain of the presequence consisting of a non-polar region, for example the 70 kDalton Outer Membrane Protein (Hase *et al.*, 1984) and porin (Freitag *et al.,* 1982) (Table 1.2). This hydrophobic region is thought to prevent the precursor from being translocated into the matrix, and acts to anchor it in the outer membrane.

Precursors which are destined for the inter-membrane space or inner-membrane in most cases possess presequences which contain a long stretch of mainly uncharged amino acids adjacent to the amino terminal matrix-targeting region, for example precytochrome c peroxidase (Kaput *et al.*, 1982), cytochrome c_1 and cytochrome b_2 (Gasser *et al.,* 1982b)(Table 1.2). Deletions of this region result in the protein being located in the matrix (van Loon *et al.*, 1986), and it was originally proposed that the uncharged region acted as a "stop-transfer" signal (van Loon and Schatz, 1987). The Rieske Fe/S protein does not possess such a hydrophobic region, yet it too is targeted to the intermembrane space (Hartl *et al.,* 1986). Recent evidence suggests that intermembrane space proteins are initially targeted to the matrix and are then retargeted to the intermembrane space (Hartl *et al.,* 1987). The uncharged region is thought to act as the retargeting signal in the case of cytochrome c_1 and cytochrome b_2, and is only revealed after the matrix targeting signal has been removed by the matrix-protease. The proposed retargeting signals of these intermediate precursors resemble bacterial export signals, suggesting that they may have evolved from their prokaryotic ancestors (Romische *et al.,* 1987). The retargeting signal of the Rieske Fe/S protein is also thought to resemble its prokaryotic equivalent, as exemplified by the Fe/S protein of *Rhodopseudomonas sphaeroides* which is

also thought to not possess a cleavable signal sequence (Gabellini and Sebald, 1986).

1.3 RECOGNITION AND BINDING TO THE MITOCHONDRION

After synthesis on cytoplasmic ribosomes, mitochondrial precursor polypeptides are localized to the mitochondrial membrane where they bind in a specific manner. This post-translational targeting has been demonstrated in *in vivo* and *in vitro* systems both with newly synthesized and with renatured precursor proteins (reviewed in Douglas *et al.,* 1986). It is possible that the initial interaction with the mitochondria is between the precursor and the lipid bilayer (Roise *et al.,* 1986). However in order to acheive the specificity and efficiency of targeting, it is thought that cytosolic recognition factors and/or mitochondrial receptors may be involved in the recognition of mitochondrial precursor proteins. Several putative components have been identified already, and their possible roles in the targeting pathway have been postulated.

1.3.1 Cytosolic Recognition Factors

Although the existence of cytosolic recognition factors for mitochondrial localization has been proposed (Bernstein *et al.,* 1989), there is no direct evidence for their existence. However, there is evidence for components which stimulate import, and as their function has not yet been clarified, it is possible that these factors may be involved in recognition.

Evidence for the requirement of additional factors for mitochondrial import was obtained using purified precursor protein and isolated rat liver mitochondria in an *in vitro* system. The efficiency of import of human preornithine transcarbamylase (pre-OTC) was improved by adding back fractions of rabbit reticulocyte lysate (Argan *et al.,* 1983). This "import factor" was shown to be proteinaceous, as its effect was abolished by preincubation with proteases, or by heat-treatment. Subsequent partial purification of this import factor showed that it formed a complex with pre-OTC, and that it was only required for the initial binding step but that it did not itself bind to

mitochondria (Argan and Lusty, 1985). Using a similar approach, Ohta and Schatz (1984) demonstrated the requirement of a soluble cytoplasmic protein of molecular weight 40 kDa from reticulocyte lysate for the increased efficiency of import of purified yeast β-subunit precursor into isolated yeast mitochondria (Ohta and Schatz 1984). A more detailed examination of the role of an import factor in binding was carried out by studying the import of the ADP/ATP translocator into *N. crassa* mitochondria (Pfanner and Neupert, 1987). In this system, a cytosolic "cofactor" in rabbit reticulocyte lysate was shown to be involved in the specific interaction of precursor with mitochondria, in that an absence of cofactor led to a reduced level of specific binding of precursor to de-energized mitochondria (Pfanner and Neupert, 1987).

As well as proteinaceous factors, an RNA species has also been shown to be required for the import of pre-OTC by demonstrating that RNase A-treated rabbit reticulocyte lysate was unable to stimulate import into isolated mitochondria (Firgaira *et al.,* 1984). Also, Pfanner and Neupert (1987) observed a reduction in the import of subunit 2 of the F_1 ATPase of *N. crassa*, but not of ADP/ATP carrier after pretreatment of reticulocyte lysate with RNase A. This suggests that there may be a ribonucleoprotein complex involved in the recognition and binding of mitochondrial precursors.

It is thought that cytosolic components may function by recognizing some common feature of presequence structure and perhaps catalyze the subsequent binding to the mitochondrial membrane (Bernstein *et al.,* 1989). However, it is possible that some of the import factors described above do not promote localization by recognition of the presequence but by maintaining the precursor protein in an import competent state, and this aspect is dicussed in Section 1.4.1.1

1.3.2 Receptors

A major step in the localization of precursor polypeptides to the mitochondria is its binding to the outer membrane and this has been shown

to occur *in vitro* (Riezman *et al.*, 1983). More detailed studies on the binding of several mitochondrial precursors have been carried out by lowering the temperature of *in vitro* import assays, which results in the accumulation of precursors on the mitochondrial surface (Pfanner and Neupert, 1987). Specific binding can be differentiated from non-specific binding by extraction of the precursor with sodium carbonate (Pfanner and Neupert, 1987).

The observed binding may be a result of the precursor protein interacting either with the lipid bilayer; or with proteins in the membrane, or both. However, in order for the mitochondrial precursor protein or complex to differentiate between the various intracellular membranes it would be more likely that there is a receptor on the mitochondrial outer membrane which could recognize either the presequence or a cytosolic recognition factor complex.

The existence of proteinaceous receptors has been postulated based on three lines of evidence. Firstly, the pretreatment of isolated yeast mitochondria with protease leads to the abolition of mitochondrial import (Zwizinski *et al.*, 1984). Secondly, antisera raised against proteins of the mitochondrial membrane have been shown to inhibit import (Ohba and Schatz, 1987). Thirdly, synthetic peptides which correspond to presequences of a mitochondrial precursor specifically inhibit the binding of some mitochondrial precursors to isolated mitochondria (Gillespie *et al.,* 1985; Chu *et al.,* 1989), possibly by blocking access to one or more components of the import apparatus.

More detailed work on binding using the mitochondrial outer membrane protein porin demonstrated the existence of high affinity binding sites for porin, and that binding to these sites was saturable (Pfaller and Neupert, 1987). The number of receptors present on the outer membrane is unknown. However porin also appeared to compete with the specific binding of the precursor of the ADP/ATP translocator, suggesting the existence of a common binding site for these two proteins (Pfaller and Neupert, 1987). Binding has also been demonstrated with the mitochondrial protein

apocytochrome c (Hennig *et al.,* 1983). These binding sites seem to differ from those for porin and ADP/ATP translocator as no protease sensitive receptors on the mitochondrial outer membrane have been identified for this protein. The receptor for F_1 β-subunit is not sensitive to elastase whereas receptor sites for other precursors appear to be. It is probable therefore that several different receptors exist, each capable of binding a different set of precursors (Sollner *et al.,* 1989). One such receptor protein has been partially purified, and has been shown to bind the precursor form of ornithine aminotransferase *in vitro* (Ono and Tuboi, 1985). A 42kDa outer membrane protein has been implicated in binding as antisera raised against it inhibits binding (Ohba and Schatz, 1987). Similarly another receptor (MOM19) has been identified using antibodies and has been isolated from the mitochondrial outer membrane, and the range of precursors to which it binds has been determined (Sollner *et al.,* 1989).

The binding of ADP/ATP carrier to mitochondria has been demonstrated to occur in two sequential steps. After the initial binding to the protease-sensitive receptor, it is thought that this precursor interacts with a second component in the mitochondrial membrane which is not sensitive to external protease. Similarly, porin shows a 2-stage binding, and it has been shown that porin can compete for binding with ADP/ATP carrier to the second binding site (Pfanner *et al.,* 1988). As porin seems to be able to compete for binding with other precursors, it has been proposed that it is the second binding site which may be common to these precursors (Pfaller *et al.,* 1988). Cytochrome c is thought to bind cytochrome c haem lyase (which is embedded in the mitochondrial outer membrane) and in this way appears to bypass the requirement for both an outer membrane receptor and a secondary receptor (Stuart *et al.,* 1990).

How might receptors function in the import process? One possibility is that a receptor recognizes a specific feature of the presequence. However it is possible that the presequence is recognized by a cytosolic factor which is itself recognized by the protease sensitive receptor on the mitochondrial

outer surface (Bernstein *et al.,* 1989). The precursor may then be able to interact with the proposed second binding site to initiate the translocation process, or may insert into the lipid bilayer directly before translocation across the membrane.

1.3.3 Energy for Binding.

As mitochondrial protein import is known to be an active process (Gasser *et al.,* 1982a; Section 1.4.4), several studies have investigated the role of a membrane potential and of ATP in the binding process. It appears that precursors fall into two categories. Precursors without a cleavable presequence such as porin (Freitag *et al.,* 1982), apocytochrome c and ADP/ATP carrier (Pfanner and Neupert, 1985), as well as precursors such as cytochrome b_2 (Daum *et al.,* 1982) and F_0 ATPase subunit 9 (Pfanner *et al.,* 1987c) have all been shown to bind to mitochondria in the absence of a membrane potential. However, other proteins do not appear to bind in a specific manner in the absence of a membrane potential, such as F_1 β-subunit (Zwizinski *et al.,* 1984) and Fe-S protein of the bc_1 complex (Hartl *et al.,* 1986).

This observed difference may reflect the variation in affinity in binding of these precursors due to the presence or absence of hydrophobic regions in the mature part of the protein (Pfanner *et al.,* 1987c). It has been demonstrated that precursors which have a hydrophobic region bind with higher affinity, and that these proteins are those which do not require a membrane potential for binding (Pfanner *et al.,* 1987c).

The requirement for ATP in binding has not been demonstrated. The depletion of ATP in an *in vitro* assay leads to the accumulation of precursors at the mitochondrial outer surface suggesting that they are still able to bind under these conditions (Pfanner *et al.,* 1987d).

1.3.4 Recognition in Other Targeting Pathways.

1.3.4.1 Signal Recognition Particle.

One of the best characterized protein targeting pathways is that of protein secretion in higher eukaryotes. The involvement of proteinaceous and RNA components in the recognition of precursors is now well understood in this process, and the possibility remains that mitochondrial protein targeting may share some features in common with this system.

Proteins which are to be targeted to the endoplasmic reticulum (ER) in the eukaryotic cell are synthesized on free cytosolic ribosomes with an amino terminal signal sequence. It is now understood that as the nascent amino terminus emerges from the ribosome, it is recognized by a ribonucleoprotein complex known as signal recognition particle (SRP)(Walter and Blobel, 1980). One of the subunits of SRP (SRP54) has been shown by cross-linking experiments to bind to the signal sequence of the nascent polypeptide (Krieg *et al.,* 1986), and the SRP complex also binds to the ribosome. Further synthesis is stopped or retarded until the complex interacts with a "docking protein", or SRP receptor, on the ER membrane. SRP is thought to dissociate from the signal sequence and the ribosome in a GTP-dependent manner (Connolly and Gilmore, 1989), and the signal sequence is thought to interact with a secondary receptor on the ER membrane (Wiedmann *et al.,* 1987; Walter, 1987). Elongation of the polypeptide continues, and is coupled to the transfer of the protein across the ER membrane.

Although an SRP-like component has not been implicated in mitochondrial targeting, mitochondrially-associated ribosomes (Kellems *et al.,*1975) have been shown to be enriched for some mitochondrial precursor mRNAs, (Suissa and Schatz, 1982) suggesting that the location of synthesis of these precursors may have an effect on their efficiency of interaction with the mitochondria *in vivo*.

1.3.4.2 Recognition in Bacterial Export.

In the export of proteins from *E. coli*, some means of recognition of the signal sequence has been postulated (reviewed in Randall *et al.,* 1987). It is thought that the product of the *secA* gene interacts specifically with the signal sequence of exported proteins (Fikes and Bassford, 1989). The 101 kDa SecA protein is thought to be located on the cytosolic face of the bacterial inner membrane (Schmidt *et al.,* 1988). After release from SecA in an ATP-dependent manner, the precursor polypeptide is thought to interact with a 49 kDa integral membrane protein, SecY (the product of the *secY/prlA* gene; Ito, 1984). It is possible that this mechanism is similar to the 2-step binding for mitochondrial targeting proposed by Pfanner *et al.* (1988).

1.3.4.3 Recognition in Chloroplast Targeting.

Protein targeting to chloroplasts is thought to resemble mitochondrial protein targeting more closely than eukaryotic secretion or protein export in bacteria. The presequences which are involved in the localization of chloroplast proteins are very similar to mitochondrial targeting sequences in many respects (Section 1.2.3.1). In terms of recognition, it has been demonstrated that chloroplast presequences can be targeted to yeast mitochondria *in vivo* and *in vitro* (Hurt *et al.,* 1986), suggesting that some component of the mitochondrial targeting machinery can recognize chloroplast targeting sequences, albeit at low efficiency. This also suggests that similar means of recognition may exist for these two organelles, whether it is cytosolic or receptor-mediated. In plants, where both chloroplasts and mitochondria exist within the same cell, the specificity of recognition is more crucial if proteins are to be correctly and efficiently targeted. Using transgenic plants, it has been demonstrated *in vivo* that recognition is very specific for the two organelles (Boutry *et al.,* 1987).

A receptor for chloroplast precursor proteins has been identified on the chloroplast envelope (Cornwall and Keegstra, 1987). Another receptor of 30kDa was identified using anti-idiotypic antibodies against SSU precursor

(Pain *et al.,* 1988), and it is possible that these receptors share significant similarity with mitochondrial receptors. However it has been demonstrated that binding to chloroplast receptors is dependent on ATP (Keegstra, 1989), and in this respect clearly differs from what is known about mitochondrial receptors.

1.4 TRANSLOCATION

1.4.1 Translocation Competence

As discussed in Section 1.2.3.2, it is thought that mitochondrial precursor proteins must be in a non-rigid conformation in order to be translocated. Translocation competence may be inherent within the protein due to the presence of the presequence or may be triggered by an interaction with the membrane or membrane receptor (Wickner, 1979). However it has been proposed that there may be accessory factors which keep the precursor protein in an import-competent (unfolded) state (Ellis, 1987); or actively participate in its unfolding (Rothman and Kornberg, 1986) either in the cytosol or at the mitochondrial surface.

1.4.1.1 Cytosolic Molecular Chaperones

Most proteins are thought to fold in a complex series of energy-requiring steps which result in a stably-folded protein. The energy required to unfold such a protein to a non-rigid conformation suitable for translocation would therefore be very high. It is thought that factors in the cell can prevent precursor proteins from folding at an early stage and also participate in the unfolding of misfolded proteins. Molecules which may be involved in these events have been termed polypeptide chain binding (PCB) proteins or "molecular chaperones", and were originally defined as components which enabled oligomers to assemble in the correct manner (Ellis, 1987; Hemmingsen *et al.,* 1988). The most well characterized chaperones are GroEL from *E.coli* (Hemmingsen *et al.,* 1988), Rubisco subunit binding protein from chloroplasts (Ellis and van der Vies, 1988), the 60kDa heat

shock protein (hsp60) from the mitochondria of *S. cerevisiae* (Cheng *et al.,* 1989) and the immunoglobulin heavy chain binding protein (BiP) from the lumen of the ER (Bole *et al.,* 1986). The term has now been extended to cover the heat-shock class of molecular chaperones which are thought to unfold misfolded polypeptides in an ATP-dependent manner (Pelham, 1986; Rothman and Kornberg, 1986) and also factors which stably maintain proteins in an early stage of their folding pathway.

Several molecular chaperones have been identified as having a role in protein translocation. In the yeast *S. cerevisiae,* four heat shock proteins of the hsp70 class encoded by the genes *SSA1, SSA2, SSA3* and *SSA4* have been shown to be required for protein translocation into the mitochondria *in vivo* and *in vitro* (Deshaies *et al.,* 1988). These proteins are also involved in protein secretion (Deshaies *et al.,* 1988; Chirico *et al.,* 1988). In higher eukaryotes, hsp70 has been shown to be involved in protein translocation into dog pancreas microsomes (Zimmermann *et al.,* 1988). Signal recognition particle has also been shown to have a chaperone function as well as a recognition role in the secretion pathway, as have ribosomes, as they can stabilize some bacterial proteins in an import-competent state (Crooke *et al,* 1988; Sanz and Meyer, 1989). In bacterial systems it has been shown that SecB and trigger factor which are known to be involved in the export process are capable of forming soluble complexes with precursor proteins *in vitro* (Crooke *et al.,* 1988; Collier *et al* 1988) and appear to stabilize precursors for membrane translocation, as does the heat-shock protein GroEL (Bockhareva *et al.*, 1988; Lecker *et al.,* 1989).

One of the main differences between protein secretion and mitochondrial protein targeting is that it has been shown that mitochondrial precursor polypeptides can be post-translationally translocated in an *in vitro* system whereas protein secretion was originally thought to be obligatorily co-translational. This has important implications for the proposed unfolded state of the precursor in that a nascent secreted protein would have no requirement to be unfolded due to its proximity to the ER when synthesized.

However, recently it has been shown that secretion can be post-translational with some proteins in some systems. In *E. coli*, several proteins are exported both co-translationally and post-translationally (Josefsson and Randall, 1981). In yeast prepro-α-factor, can be post-translationally translocated (Hansen *et al.,* 1986). For higher eukaryotes, the same phenomenon has been shown using fusion proteins translocated into dog pancreas microsomes (Perara *et al.,* 1986). However, in the latter case, SRP and ribosomes are still required.

An SRP-like factor has not been identified biochemically in *S. cerevisiae*, and it is possible that some lower eukaryotes can bypass the requirement for elongation arrest and co-translational targeting by using an additional mechanism for maintaining import-competence such as the hsp70 proteins or other chaperones. As hsp70 proteins have been shown to be required *in vivo* for secretion as well as for mitochondrial targeting, it is possible that there may be a common mechanism for maintaining both groups of precursors in an import competent state (Deshaies *et al.,* 1988).

How then do these chaperones specifically bind to precursor proteins? There have been no studies on the specifity of chaperones for mitochondrial precursors. However, it has been shown that hsp70 and BiP have a higher affinity for some proteins than others (Flynn *et al.*, 1989). Studies in *E. coli* suggest that chaperones do not recognize leader sequences, as the SecB protein recognizes the mature part of maltose-binding protein (Collier *et al.,* 1988). Also SecB, trigger factor and GroEL all form complexes with the mature OmpA protein but do not form complexes with globular proteins such as ovalbumin (Lecker *et al.,* 1989). It has been proposed that all nascent polypeptides form complexes with chaperones but that cytoplasmic proteins can fold rapidly enough so that the chaperone is displaced (Wickner, 1989). In this model, only exported proteins form stable complexes with the chaperone, and this allows them to interact with other components of the targeting apparatus.

1.4.1.2 ATP Requirement for Import-Competence.

It has been suggested that a mitochondrial precursor must be in a loosely-folded conformation in order to be translocated (Section 1.2.3.2). The role of ATP in the proposed unfolding of a precursor protein in translocation was first demonstrated in the *in vitro* insertion of M13 procoat protein into microsomes, a process which also requires ATP (Wiech *et al.,* 1987). In this system, the presence of ATP led to the precursor protein being more sensitive to added proteases, suggesting that it may be prevented from folding due to the ATP.

In the translocation of mitochondrial precursors, a reduction in the amount of ATP or GTP in an *in vitro* import assay led to the accumulation of precursors at the mitochondrial surface, suggesting that the translocation step had been affected (Pfanner *et al.,*1987d). ATP hydrolysis was required for import to continue. More specific evidence for the role of ATP in the unfolding of mitochondrial precursors was shown *in vitro* using incompletely synthesized precursor chains (Verner and Schatz, 1987). These nascent precursors were shown to be in an incompletely folded state, and their *in vitro* import into mitochondria was shown to be independent of added ATP, whereas the import of the completed precursor is dependent on ATP (Verner and Schatz, 1987).

How might ATP be involved in the unfolding of precursors? As discussed in the previous section, chaperones are thought to be involved in the unfolding process. The Hsp70 group of heat shock proteins are known to have an ATPase activity (Pelham, 1986) and their involvement in the unfolding of mitochondrial precursors fits in with the requirement for ATP.

1.4.2 Contact Sites.

The site of translocation of proteins across mitochondrial membranes has been proposed to lie at regions where the inner and outer mitochondrial membranes appear to converge (Kellems *et al.,* 1975). Evidence for this was obtained more recently when mitochondrial precursors were shown to

accumulate at these contact sites (Schleyer and Neupert, 1985). Artificial trapping of polypeptides at these sites was acheived by preincubation of the mitochondrial precursor with antibodies which recognized the carboxy terminus of the protein, and which presumably blocked translocation across the membranes. Also, low temperature and low levels of NTPs can cause precursors to accumulate at contact sites (Pfanner *et al.,* 1987a). Similarly, a fusion protein of preCOXIV-DHFR-BPTI (Bovine Pancreatic Trypsin Inhibitor) is blocked at contact sites (Vestweber *et al.,* 1989). In each case, the precursor has been shown to span both membranes as it is accessible to the matrix protease at the amino terminus, yet at the same time is sensitive to externally added proteases at regions of the carboxy terminus (Hartl *et al.,* 1989).

What is the role of these contact sites in translocation? As they seem to be stable structures (Schwaiger *et al.,* 1987), it has been suggested that proteins are involved in conferring this stability, and that these proteins might also be directly involved in the translocation process (Pfanner *et al.,* 1988). This aspect is discussed further in the next section (1.4.3).

Contact sites between the outer and inner membranes of the chloroplast envelope have also been identified, and one of the proposed chloroplast receptors (30kDa SSU receptor) has been demonstrated to be located at these sites (Pain *et al.,* 1988). Bacterial contact sites or zones of adhesion (Bayer and Thurow, 1977) are also thought to be involved in translocation of exported proteins (Randall *et al.,* 1987)

1.4.3 Translocation Components.

Although it has been proposed that translocation of a precursor polypeptide might occur through the lipid bilayer directly as in the helical hairpin hypothesis (Engelman and Steitz, 1981), it is more likely that an integral membrane protein complex mediates translocation of mitochondrial precursors (Singer *et al.,* 1987). Support for this theory has been obtained from the observation that precursors trapped in contact sites are accessible to

aqueous perturbants, and are therefore not embedded in the lipid bilayer. (Pfanner *et al.,* 1987a). A proteinaceous apparatus would provide such a hydrophilic environment, and this may take the form of a pore through which the precursor polypeptide can pass in an unfolded state.

The photocrosslinking of a trapped precursor fusion has led to the identification of a protein component which is thought to be present in contact sites (Vestweber *et al.,* 1989). This 42 kDa protein (ISP42 - Import Site Protein) has been shown to be located in the mitochondrial outer membrane, and suprisingly is not enriched at contact sites. To explain this phenomenon, it has been proposed that there is a dynamic interaction between ISP42 and other components of the translocation site during the translocation process. The possibility exists that components of the translocation apparatus are also involved in the binding of the precursor polypeptide, either directly or at the secondary binding step which has been observed with the 2-step import of ADP/ATP carrier (Pfanner and Neupert, 1987). It is also possible that the processing step on the inside of the inner mitochondrial membrane may be involved in the vectorial transfer of the precursor polypeptide (Section 1.5).

In other targeting systems, the existence of proteinaceous translocation components has been proposed. It is thought that a protein pore may be involved in protein secretion across the endoplasmic reticulum (Gilmore and Blobel, 1985; Evans *et al.,* 1986). Similarly, in *E. coli* the product of the *secY* (*prlA*) gene has been shown to be an integral membrane protein (Akiyama and Ito, 1985), and genetic evidence implicates it in the targeting process (Emr *et al.,* 1981). In chloroplasts, ATP-dependent phosphorylation of a 51 kDa envelope protein of chloroplasts correlates with translocation (Hinz and Flugge, 1988) and a 30 kDa receptor has been shown to be located at contact site (Pain *et al.,* 1988).

1.4.4 Energy requirement for Translocation.

The ability to perform mitochondrial import experiments *in vitro* after the synthesis of the mitochondrial precursor enabled several aspects of targeting to be dissected. It was initially demonstrated that import was energy-dependent in an *in vitro* system (Gasser *et al.,* 1982a), and that this energy was provided by nucleoside triphosphates as well as an electrochemical potential (Pfanner and Neupert, 1986). It was later shown that hydrolysis of a phosphodiester bond in either ATP or GTP occurred outside the mitochondria (Chen and Douglas, 1987). This was confirmed by further experiments demonstrating the requirement for ATP or GTP for translocation (Eilers, *et al.*, 1987; Pfanner *et al.,* 1987d).

The requirement for ATP has been observed in other protein targeting systems and may represent a general phenomenon. The translocation of proteins across the plasma membrane in *Escherichia coli* has been shown to require ATP (Chen and Tai, 1985). Prepro-α-factor translocation across the endoplasmic reticulum of yeast has been shown to be ATP-dependent *in vitro* (Hansen, *et al.*, 1986); and the requirement for ATP has been shown for protein translocation into chloroplasts (Grossman *et al.,* 1980; Flugge and Hinz, 1986). In this case, the ATP requirement for translocation has been shown to be internal to the chloroplast.

What is the function of the nucleoside triphosphate required for translocation? It is possible that ATP may be required to provide energy for a putative transporter protein ("protein-translocating ATPase") which spans the mitochondrial membrane(s), thus participating in the import process directly (Chen and Douglas, 1987; Section 1.4.3). Also, the ATP may be required to phosphorylate a component of the import machinery. However, the fact that high levels of ATP are required for complete insertion into the mitochondrial outer membrane led to the proposal that ATP is required for maintaining the precursor in an import-competent state as has been previously discussed (Section 1.4.1.2; Pfanner *et al* , 1987d).

During preliminary work with yeast sphaeroplasts, it was demonstrated that the addition of an uncoupler of oxidative phosphorylation such as carbonylcyanide *m*-chlorophenylhydrazone (CCCP) prevented the processing of some mitochondrial precursors (Nelson and Schatz, 1979). It was later shown that these precursors accumulate outside the mitochondria in the presence of CCCP (Reid and Schatz, 1982a) and that the lack of processing was due to a block in the translocation step rather than at the processing step (Reid and Schatz, 1982b). It has been demonstrated using isolated mitochondria and the ATP hydrolysis inhibitor oligomycin that import requires an energized membrane (Schleyer, *et al.*, 1982; Gasser *et al.*, 1982a). These findings show similarity to the energy requirement for the export of proteins across the bacterial plasma membrane which also requires a membrane potential as well as ATP (Chen and Tai, 1985).

There are several suggestions as to the possible use of a membrane potential in translocation. Energy in the form of a chemical potential, ΔpH, may be required to drive the part of the protein translocating machinery itself, such as a proton-driven translocase (Section 1.4.3). However, evidence suggests that it is the electrical component, $\Delta\psi$, of the total motive force which is required (Pfanner and Neupert, 1985). Therefore it may be an electrophoretic effect which drives the positively-charged regions of the precursor into the membrane as the initial stage in translocation (Pfanner and Neupert, 1985). The eventual identification of the components of the protein translocating machinery may reveal the precise nature of the requirement for energy.

1.5 PROCESSING.

Most proteins that are targeted to the mitochondria are cleaved before they are assembled into functional complexes. Cleavage of many proteins has been shown to be catalyzed by a soluble metallo-endoprotease which is located in the mitochondrial matrix (McAda and Douglas, 1982; Bohni *et al.*, 1983; Kumamoto *et al.*, 1986) and which is thought to recognize some structural domain of the presequence (Section 1.2.4).

Purification of this protease from *N. crassa* (Hawlitschek *et al.,* 1988) has revealed that processing activity consists of two proteins, mitochondrial processing peptidase (MPP) and processing enhancing protein (PEP). MPP is a monomer of 57 kDa which is soluble in the mitochondrial matrix, and has a small amount of processing activity on its own. PEP is also a monomeric protein 52 kDa which has no apparent activity on its own, is partly associated with the inner membrane, and is 15-fold more abundant than MPP. It is thought that MPP contains the catalytic site and that PEP stimulates the processing activity of MPP (Hawlitschek *et al.,* 1988).

Two temperature sensitive yeast mutants (*mas1* and *mas2*) which are defective in processing of mitochondrial precursor polypeptides have been isolated (Yaffe and Schatz, 1984). The cloning of the wild-type *MAS1* and *MAS2* genes (Witte *et al.,* 1988; Jensen and Yaffe, 1988) has revealed that they encode the two components of the processing peptidase MPP and PEP respectively, and that these components are structurally related (Jensen and Yaffe, 1988; Pollock *et al.,* 1988). The *mas2* mutant appears to accumulate precursor proteins outside the mitochondrial inner membrane at the non-permissive temperature (Yaffe and Schatz, 1984). There are two possibilities to account for this observation. Either the processing protease components may have another function in addition to processing, for example it has been suggested that the PEP component may participate in the translocation step (Hawlitschek *et al.,* 1988). Or that translocation and processing are tightly coupled so that at the non-permissive temperature a block in processing would lead to a back-log at the exit from the translocation machinery. However, processing is not essential for translocation as precursors can be accumulated in the matrix under some circumstances (Hartl *et al.,* 1986).

The fact that the *mas* mutants are lethal at the non-permissive temperature suggests that either the accumulation of precursors with presequences may disturb mitochondrial membrane functions, or that the import of an essential mitochondrial protein is blocked in these mutants. Both these explanations suggest that some mitochondrial function is essential

even when the yeast cell is growing fermentatively (Kovacova *et al.,* 1968). However, the cleavage event is not always necessary for the correct assembly of the protein into a functional complex as has been demonstrated in the case of the β-subunit of the F_1ATPase. (Vassarotti *et al.*, 1987a).

Comparison of the mitochondrial matrix peptidase to the leader peptidase of bacteria (Zwizinski and Wickner, 1980) or to the eukaryotic signal peptidase of the endoplasmic reticulum (Evans *et al.,* 1986) reveals no similarity either in terms of structure or in terms of recognition of cleavage site. It is more likely that the mitochondrial matrix protease is similar to the 180 kDa stromal peptidase of chloroplasts (Robinson and Ellis, 1984). However, similarities in recognition have not been demonstrated.

As discussed in Section 1.2.4, some precursors which are destined for the intermembrane space or inner membrane are processed by a second protease (Daum *et al.,* 1982). In some cases, this second processing event is also carried out by the matrix peptidase (Schmidt *et al.,* 1984). Other intermediate precursors are processed by a peptidase which is located in the intermembrane space (Hartl *et al.,* 1987). This peptidase is as yet uncharacterized.

1.6 INTRAMITOCHONDRIAL SORTING.

Several mitochondrial precursors are thought to possess retargeting signals which direct them out of the matrix to their final destination in the inner membrane or intermembrane space (Section 1.2.5). In this respect, the second targeting step may be analogous to protein export in *E. coli*, from which the pathway may have evolved. Evidence for the conservative sorting of cytochrome c_1 and the Rieske Fe/S protein has been obtained by examining the bacterial equivalents of these proteins (Hartl *et al.,* 1986; Hartl *et al.,* 1987) which are normally exported across the plasma membrane. The leader peptides of these proteins are similar to the intermediate presequence of their mitochondrial equivalents (Romische *et al.,* 1987)

It is thought that several pathways exist for retargeting within the mitochondria, and that specific protein components may be involved which interact with the retargeting sequence just as recognition factors and molecular chaperones are thought to be involved in bacterial export. One protein which is thought to be involved in the re-export pathway is Hsp60, as yeast mutants which are defective in Hsp60 accumulate the intermediate form of cytochrome b_2 (Cheng *et al.,* 1989). This protein is homologous to the GroEL protein which may affect export of bacterial proteins (Lecker *et al.,* 1989).

1.7 ASSEMBLY OF MITOCHONDRIAL SUBUNITS.

As it is thought that mitochondrial precursors are unfolded in order to be translocated, then it follows that they need to refold once they reach their final destination in the mitochondria. Subsequently many mitochondrial proteins assemble into multi-subunit complexes and some proteins require the addition of a prosthetic group for function eg haem attachment.

These folding and assembly processes were predicted to involve other factors, in a similar way that factors are thought to be involved in the unfolding process (Section 1.4.1.1). The involvement of one such assembly factor (Hsp60) has been established genetically in yeast, and a mutant defective in this protein (*mif4*) has been shown to be deficient in the assembly of β-subunit and Rieske Fe/S protein *in vivo* and *in vitro* (Cheng *et al.,* 1989). The Hsp60 protein is homologous to GroEL in *E. coli* and Rubisco subunit-binding protein in chloroplasts, both of which are known to act as molecular chaperones in the assembly of multisubunit complexes (Section 1.4.1.1).

Haem attachment has been studied in the case of apocytochrome c. The location of this step has been identified as in the mitochondrial inner membrane, facing the cytoplasmic surface (Enosawa, and Ohashi, 1986) where the enzyme cytochrome c haem lyase is located. This modification may effectively immobilize it in its correct physiological location, and may in fact play a role in the translocation of apocytochrome c (Stuart *et al.,* 1990).

Very little is known about the stage at which other mitochondrial proteins can attach prosthetic groups such as haem or flavin nucleotides, though it would be more likely for these steps to be located after all the membrane translocation steps have taken place.

1.8 THE STUDY OF TARGETING OF F_1 ATPase β-SUBUNIT TO THE MITOCHONDRIA IN *S. CEREVISIAE* AS A MODEL SYSTEM.

Although the research summarized in this Chapter has made considerable progress in resolving the mechanism of mitochondrial protein targeting, several key questions remain unanswered. Firstly, although it is known that a mitochondrial presequence carries information which is both necessary and sufficient for efficient targeting (Section 1.2.2), the precise nature of this information is not understood, although it is assumed tertiary structure is involved. Secondly, it is not known which features of a targeting sequence are involved in each step in the import pathway - for example binding or translocation. Thirdly, the nature of the interaction between the precursor and components of the targeting apparatus such as recognition factors, receptors, chaperones or translocases is not at all well understood, mainly because the nature and number of these components have not been identified.

In order to attempt to answer some of these problems, the work described in this thesis is concerned with the targeting of the precursor of the β-subunit of the F_1 portion of the mitochondrial ATP synthase (F_1 β-subunit) to the mitochondria of *S. cerevisiae*. The advantage of this system is that as well as being able to dissect the targeting process biochemically *in vitro,* and examining phenotypic effects *in vivo*, the powerful tool of genetics can also be used to look at specific interactions between the precursor and other gene products.

The mitochondrial ATP synthase consists of 10 subunits, 3 of which are mitochondrially encoded, and the remainder are nuclear encoded (Dujon, 1981). The complex serves to synthesize ATP using the proton gradient

across the mitochondrial membrane generated by the electron transport chain. Expression of many of the subunits appears to be regulated in yeast, as function is only required when the yeast is growing on non-fermentable carbon sources. The β-subunit is encoded by the *ATP2* gene, knock-out mutants of which are unable to grow on non-fermentable carbon sources such as glycerol or lactate. The *ATP2* gene has been cloned (Saltzgaber-Muller *et al.,* 1983) and its DNA sequence has been determined (Takeda *et al.,* 1986). The deduced amino acid sequence predicts a precursor protein of 509 amino acids which would have a molecular weight of 56kD (Maccechini *et al.,* 1979). In all respects, the β-subunit appears to be a typical mitochondrial precursor, with a positively charged amino terminal targeting region (Figure 1.2). The minimum region required which is sufficient to target a fusion protein to the mitochondria has been defined as the first 15 amino acids (Walker, 1987; Walker *et al.,* 1990). The precursor protein is imported to the mitochondrial matrix where it is cleaved by the matrix protease to give a protein of approximately 54kDa.

The initial experiments described in this thesis were carried out in an attempt to generate sufficient β-subunit precursor protein for biochemical studies on the interaction bewteen the precursor β-subunit and components of the targeting pathway (Chapter 3). However, deletion mutagenesis used to define regions of the β-subunit required for targeting led to a unique phenotype *in vivo* (Chapter 4). This paved the way for the genetic approach adopted in Chapter 5 which hopefully would identify components of the targeting pathway.

CHAPTER TWO

MATERIALS AND METHODS

2.1 STRAINS AND PLASMIDS

Strains of *Saccharomyces cerevisiae* used in this study are listed in Table 2.1. Strains of *Escherichia coli* used are listed in Table 2.2. Plasmid vectors used are illustrated in Figures 2.1 to 2.12. Recombinant plasmids are described in detail in the relevant section.

2.2 GROWTH AND MAINTENANCE OF STRAINS.

Strains of *E. coli* were grown in liquid culture in LB media (Section 2.3.1) with or without ampicillin (50 μg/ml) with shaking at 180 r.p.m. at 37°C unless otherwise stated. They were maintained on LB agar plates (Section 2.3.1) at 4°C and subcultured every 3 weeks. Permanent stocks were made from liquid cultures by freezing in 20% glycerol at -70°C.

Strains of *S. cerevisiae* were grown in the appropriate media (Section 2.3.2) shaking at 180 r.p.m. at 30°C unless otherwise stated. They were maintained on appropriate plates at 4°C and subcultured every 2 weeks. Permanant stocks were made from liquid culture by freezing cells in 20% glycerol at -70°C

2.3 MEDIA

2.3.1 Media for *E.coli*

LB	per litre
Yeast Extract	5 g
NaCl	5 g
Tryptone	10 g

Antibiotics were added to give a final concentration of 50 μg/ml (ampicillin) and 170 μg/ml (chloramphenicol for amplification of plasmid DNA). Plates were made by the addition of 20 g agar per litre.

Table 2.1 Strains of *S. cerevisiae.*

NAME		GENOTYPE / PLASMID	SOURCE/CONSTRUCTION
D273-10-B		Wild type	G Schatz[1]
D273-10-B-ρ^-		ρ^- mutant of D273-10-B	G Schatz[1]
SF747-19D	α	*his4 leu2-3 leu2-112 ura3-52*	R.Schekman
GR1	α	*his4 leu2-3 leu2-112 ATP2::URA3-52*	G A Reid
JQ1	α	*his4 ura3-52 atp2::LEU2*	G A Reid[2]
DBY746	α	*his3-Δ1 leu2-3 leu2-112 ura3-52 trp1*	D Jamieson
DBY747	a	*his3-Δ1 leu2-3 leu2-112 ura3-52 trp1*	D Jamieson
EMY2	α	*his4 leu2-3 leu2-112 ura3-52* /p*LEU2 PHO5::ATP2*	SF747+p402yATP2
EMY5	α	*his4 leu2-3 leu2-112 ura3-52* /p*LEU2 PGK::ATP2*	SF747+pEE3
EMY6	α	*his4 leu2-3 leu2-112 ura3-52 ATP2::URA3* /p*LEU2 PGK::ATP2*	GR1 + pEE3
EMY9	α	*his4 leu2-3 leu2-112 ura3-52* /p*LEU2*	SF747 + pMA91
EMY11	α	*his4 leu2-3 leu2-112 ura3-52* /p*URA3 ADH::ATP2*	SF747 + pEE4
EMY21	α	*his4 ura3-52 atp2::LEU2* /p*URA3 ADH:: 2-15atp2*	JQ1 + pML7Y
EMY25	a	*his3-Δ1 leu2-3 leu2-112 ura3-52 trp1 atp2::LEU2*	DBY747+pJQ10 disruption
EMY28	a	*his3-Δ1 leu2-3 leu2-112 ura3 trp1 atp2::LEU2* /p*URA3 ADH*::Δ2-15*atp2*	EMY25 + pML7y
EMY40	a/α	*his4 HIS3 leu2-3 leu2-112 ura3-52 TRP1 atp2::LEU2* /p*URA3 ADH1*::Δ2-15*atp2* *HIS4 his3-Δ1 leu2-3 leu2-112 ura3-52 trp1 ATP2*	EMY172 x DBY747
EMY42	a	*his4 leu2-3 leu2-112 ATP2 ura3-52 mts1*	Progeny of EMY40
EMY49	α	*his4 ura3-52 atp2::LEU2* /p*URA3 ADH1*::Δ16-35*atp2*	JQ1 + pEE17
EMY50	α	*his4 ura3-52 atp2::LEU2* /p*URA3 ADH1*::Δ2-35*atp2*	JQ1 + pEE19
EMY51	α	*his4 ura3-52 atp2::LEU2* /p*URA3 ADH1*::Δ16-35*atp2*	JQ1 + pEE13
EMY53	α	*his4 leu2-3 leu2-112 ATP2 ura3-52 mts1*	Progeny of EMY40
EMY54	α	*his4 ura3 atp2::LEU2 mts1*	Cured EMY172

(Table 2.1 cont.)

NAME		GENOTYPE / PLASMID	SOURCE/CONSTRUCTION
EMY55	α	*his4 ura3-52 atp2::LEU2 /pURA3 ADH1::Δ2-15atp2*	JQ1 + pEE21
EMY56	α	*his4 ura3-52 atp2::LEU2 /pURA3 ADH1::ATP2*	JQ1 + pEE4
EMY59	α	*his4 ura3-52 atp2::LEU2 /pURA3 ADH1::Δ2-35atp2*	EMY54 + pEE19
EMY61	α	*his4 ura3-52 atp2::LEU2 /pURA3 ADH1::Δ2-35atp2*	JQ1 + pEE19
EMY63	a	*his3-Δ1 leu2-3 leu2-112 ura3-52 trp1 MTS1::pEE46*	DBY747 + pEE46 integrant
EMY64	α	*his4 leu2-3 leu2-112 ura3-52 mts1::pEE46*	EMY53 + pEE46 integrant
EMY65	a/α	*his3-Δ1 HIS4 leu2-3 leu2-112 ura3-52 trp1 MTS1::pEE46* / *HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 mts1*	EMY63 x EMY53 diploid
EMY66	a/α	*his3-Δ1 HIS4 leu2-3 leu2-112 ura3-52 trp1 MTS1* / *HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 mts1::pEE46*	DBY747 x EMY64 diploid
EMY67	a/α	*his3-Δ1 HIS4 leu2-3 leu2-112 ura3-52 trp1 MTS1* / *HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 mts1*	DBY747 x EMY53 diploid
EMY68	a/α	*his3-Δ1 HIS4 leu2-3 leu2-112 ura3-52 trp1 MTS1* / *HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 mts1::pEE47*	EMY67 + pEE47 disruptant
EMY153	α	*his4 leu2-3 leu2-112 ura3-52 mts2 atp2::LEU2 /pURA3 Δ2-15atp2*	Suppressor mutant of EMY21
EMY172	α	*his4 leu2-3 leu2-112 ura3-52 mts1 atp2::LEU2 /pURA3 Δ2-15atp2*	Suppressor mutant of EMY21

References

1. Todd *et al.*,1979.

2. Walker *et al.*, 1990.

All other strains - this study.

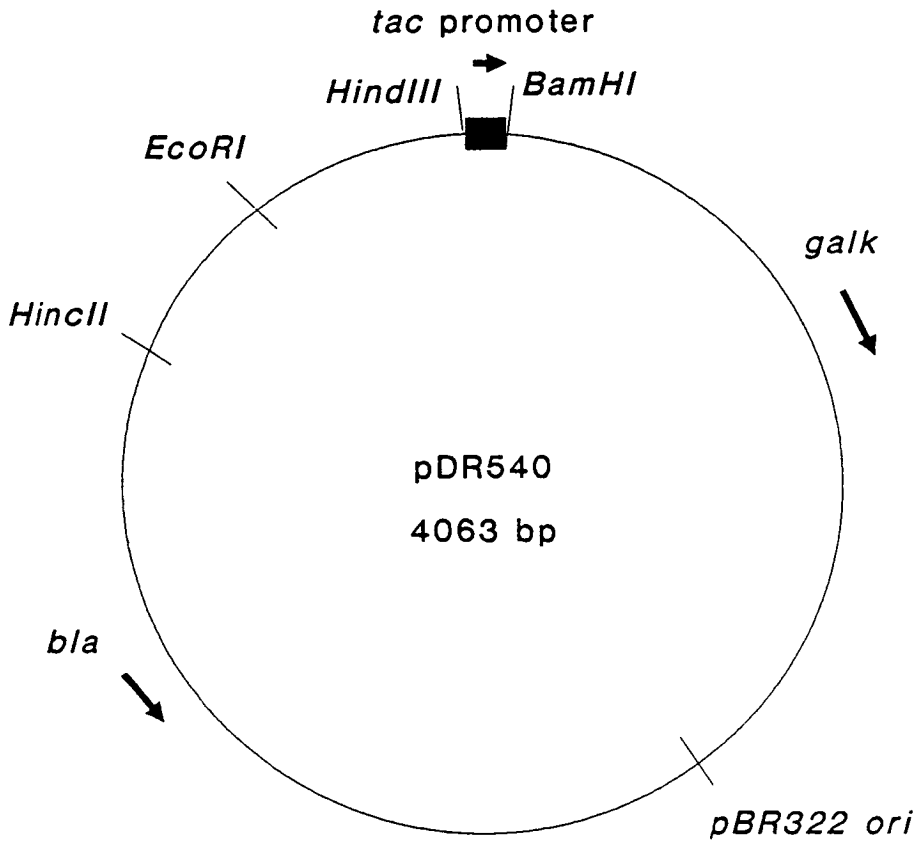
Table 2.2 Strains of *Escherichia coli.*

STRAIN	GENOTYPE	SOURCE
HB101	*leuB F⁻ hsdR⁻ hsdM⁻ recA13 ara-14 proA2 xyl-5 lacY1 galK2 rpsL20 (Sm^R) mtl-1 SupE44*	G A Reid[1]
MM294	*pro endoA thi hsdR supE44*	D. Lilley[2]
TG1	K12 Δ*(lac-proAB) supE thi hsdΔ5 /F'traD36 proA⁺B⁺ lacIᵠ lacZΔM15*	Amersham[3]
NF1	K12 Δ*H1* Δ*trp lacZ* λ *Nam7 Nam53 cI857* Δ*H1*	D.Jamieson[4]
KC1101	*lacU169 proA lon araD139 strA hflA150 chr::Tn10 /F' lacI lacZ::Tn5*	K Chapman

References

1. Boyer and Roulland-Dussoix, 1969.
2. Meselson and Yuan, 1968.
3. Gibson, 1984.
4. Stanley and Luzio, 1983.

Figure 2.1 pDR540 *tac* Promoter Vector

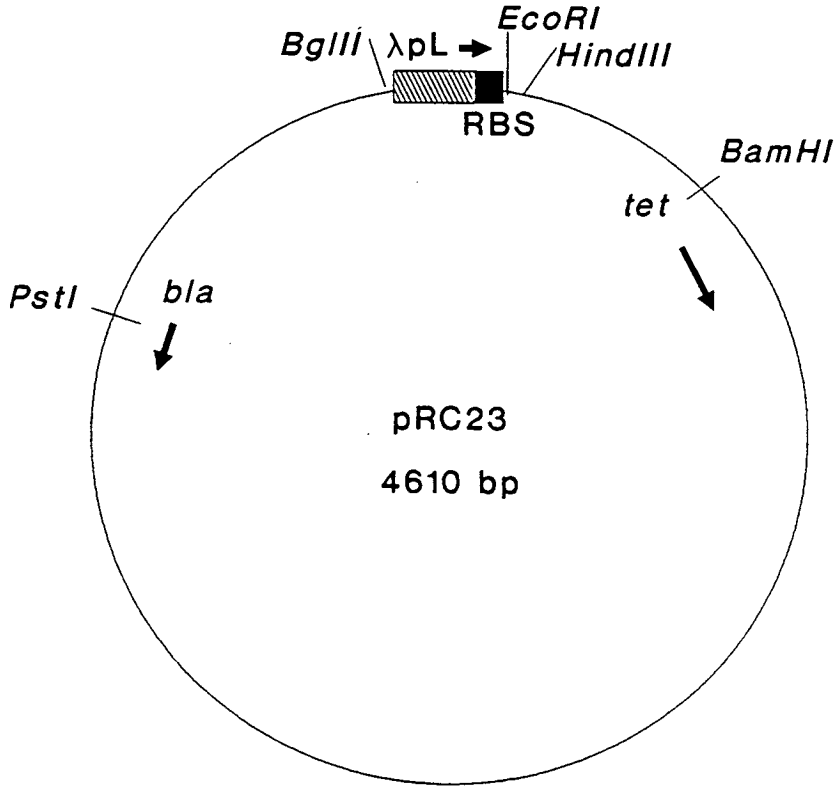


***tac* Promoter Sequence:**

```
                                                                Shine Dalgarno
5'                      -35                 -10      +1                ↓     ↓
AAGCTTACTCCCCATCCCCCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAATTTCACACAGGAAACAGGATCC
   |                    |                                                          |
HindIII              HincII                                                     BamHI 3'
```

Plasmid pDR540 contains the strong tac promoter which is a fusion of the trp and lac promoters, and also the ribosome binding site from the lac gene (Russell and Bennett, 1982). DNA fragments can be inserted at the unique BamHI site.

Figure 2.2 pRC23 λp_L Promoter Vector



Synthetic Ribosome binding site:

Shine Dalgarno

↓

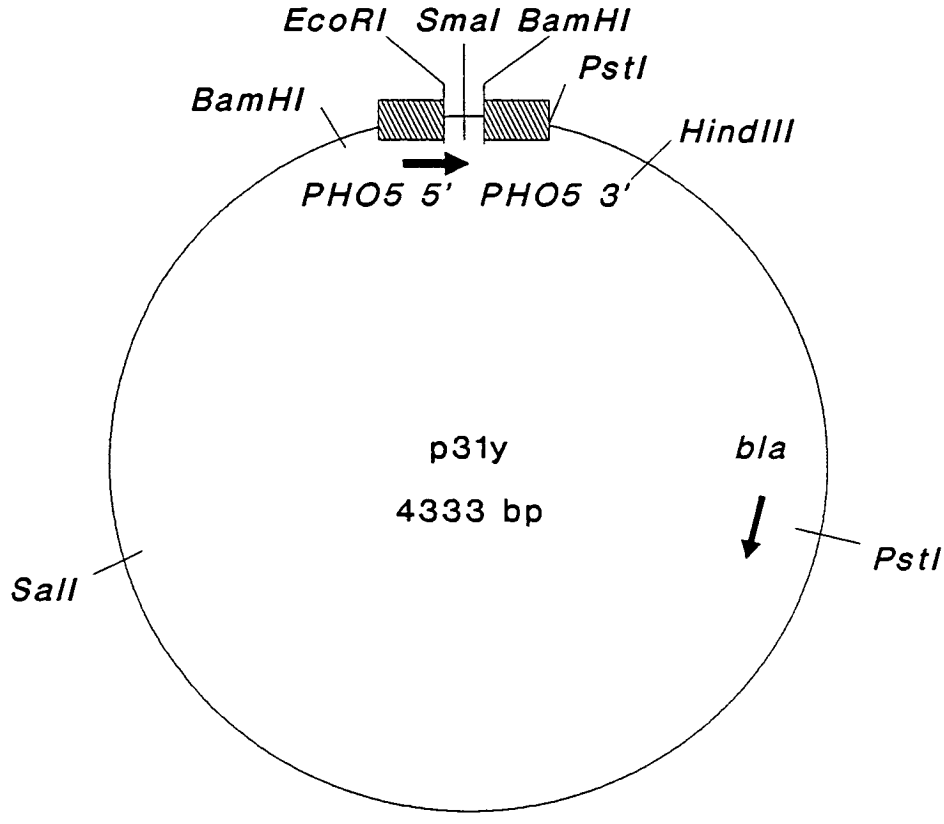
TTAAAAATTAAGGAGGAATTC

|

EcoRI

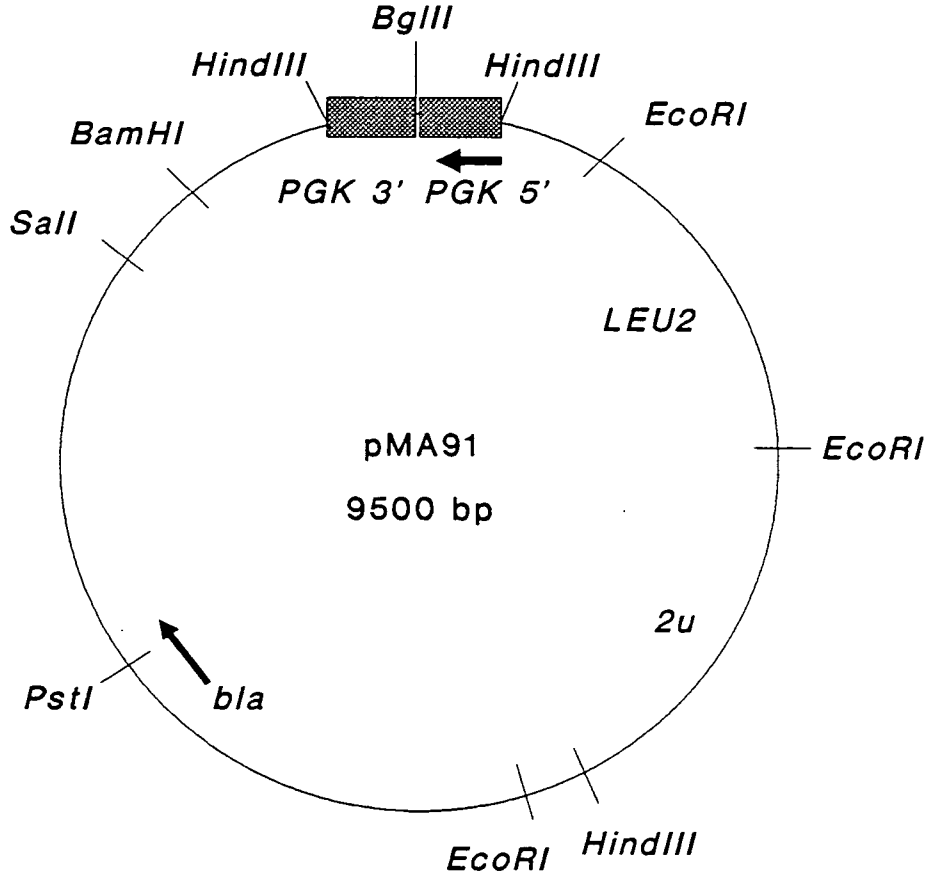
Plasmid pRC23 contains the λp_L promoter on a 250 bp BglII-HaeIII fragment adjacent to the synthetic ribosome binding (Crowl et al., 1985). Fragments of DNA to be expressed can be cloned into the unique EcoRI site.

Figure 2.3 p31y *PHO5* Promoter Vector



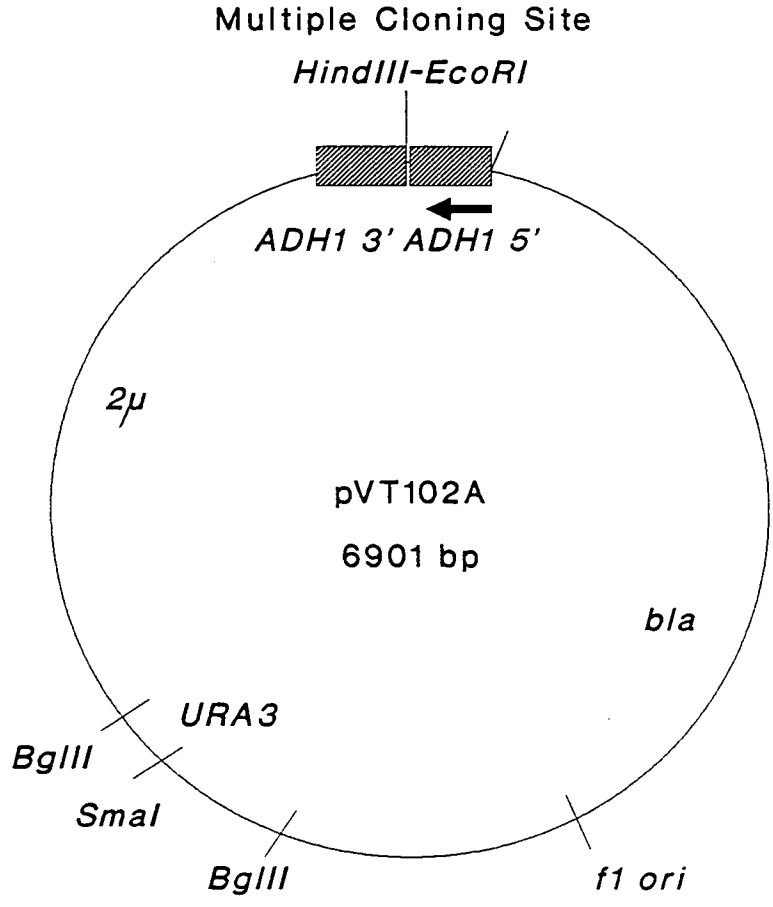
Plasmid p31y was obtained from A. Hinnen. It contains the PHO5 promoter and terminator regions, and unique SmaI and EcoRI sites for cloning fragments. It contains no yeast selectable marker or origin of replication.

Figure 2.4 pMA91 *PGK* Promoter Vector.



Plasmid pMA91 was obtained from A. Kingsman. It contains the PGK promoter and terminator regions, and 2μ origin of replication and is described by Mellor et al., (1983). DNA fragments can be cloned into the unique BglII site.

Figure 2.5 pVT102A *ADH* Promoter High Copy Vector

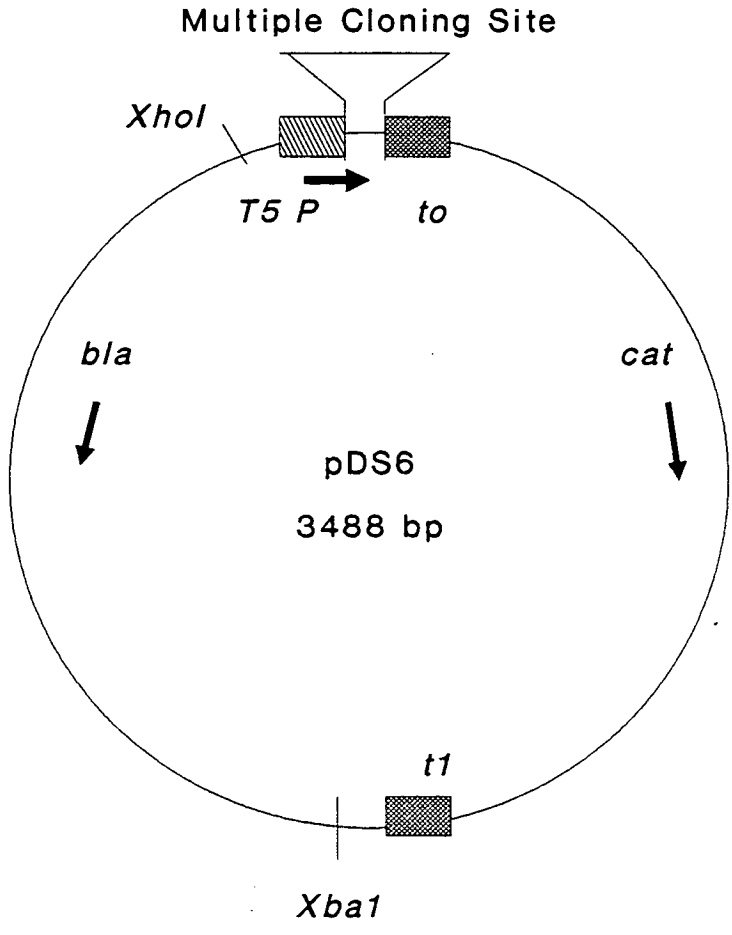


Multiple Cloning Site:

HindIII-PvuII-PstI-XhoI-SacI-XbaI-EcoRI

Plasmid pVT102A contains the ADH promoter and terminator regions, and the 2μ origin of replication. It was constructed by G Reid by filling in the EcoRI site of pVT102U (Vernet et al., 1987), and inserting EcoRI linkers into the filled in BamHI site of the multiple cloning site.

Figure 2.6 pDS6 T_5 Promoter Vector.

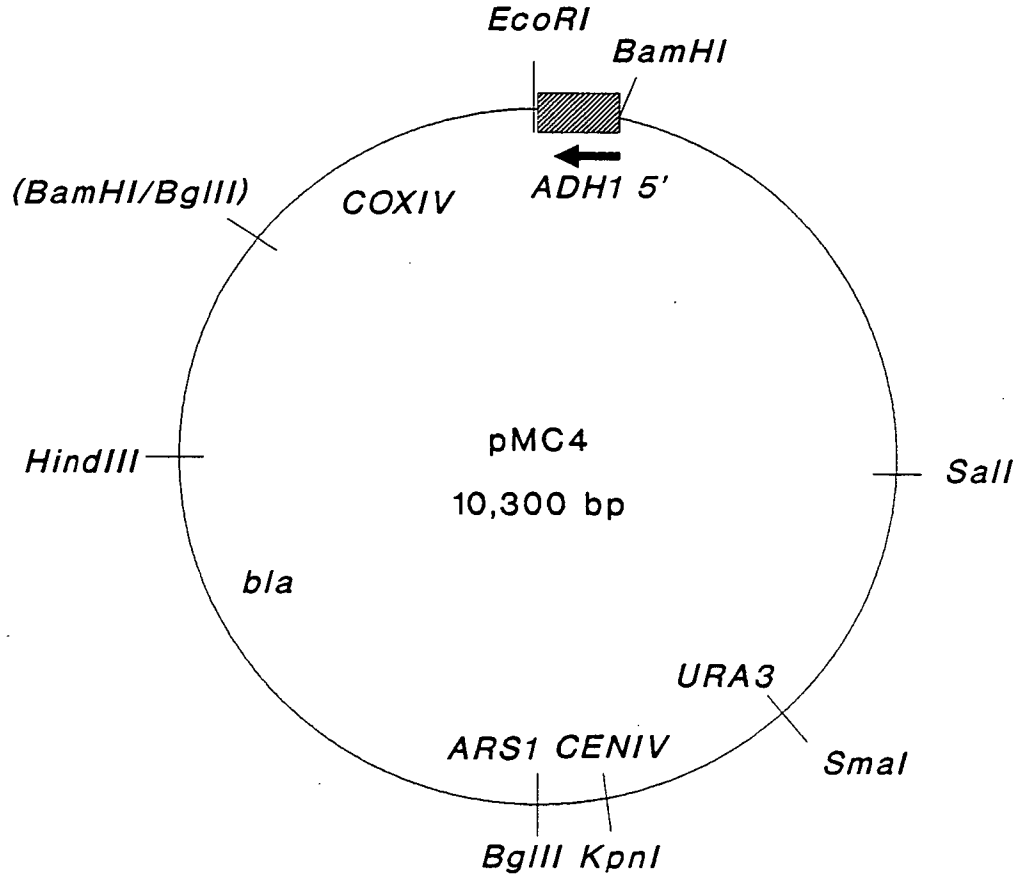


Multiple Cloning Site:

EcoRI-SmaI-BamHI-SalI-PstI-HindIII

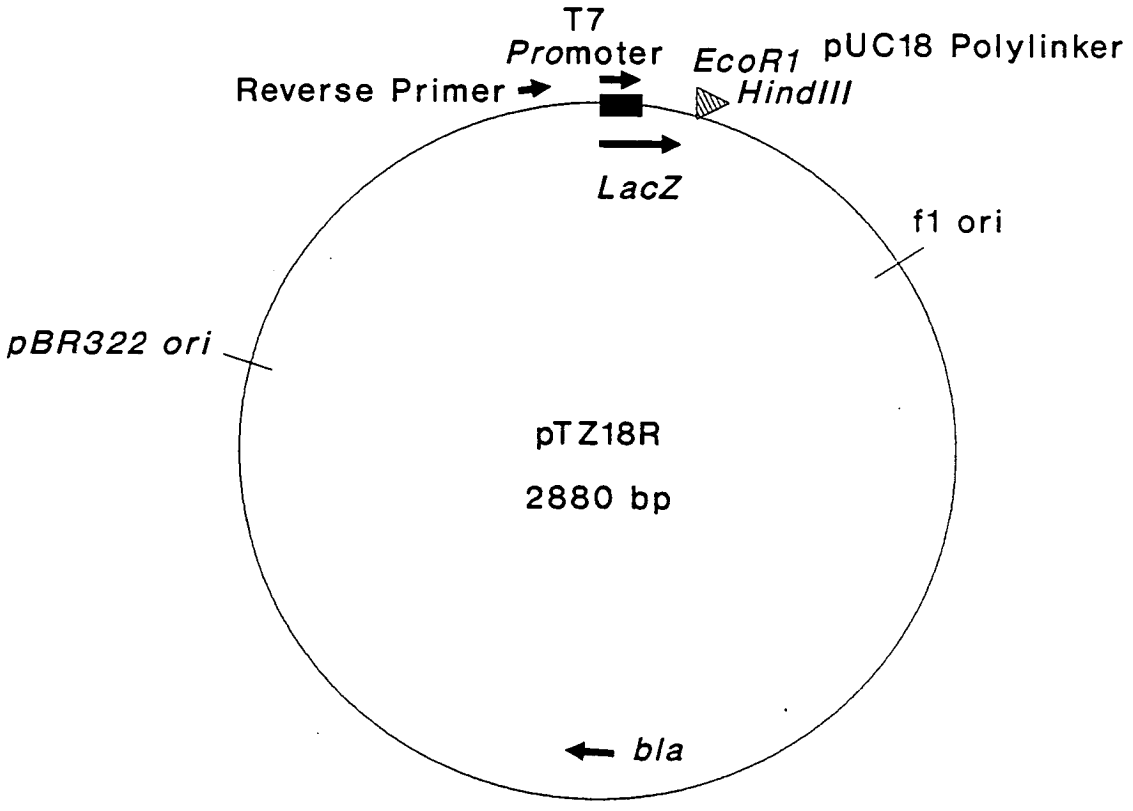
Plasmid pDS6 contains the T_5 promoter adjacent to the multiple cloning site (Stueber et al., 1984). Downstream is a transcription terminator followed by the cat gene.

Figure 2.7 pMC4 *ADH* Promoter Low Copy Vector.



Plasmid pMC4 was obtained from A. Baker and G. Schatz, and was constructed to express COXIV in yeast. It contains the ADHI promoter fused to the COXIV sequence at a unique EcoRI site, and the CYCI terminator. For selection in yeast it contains the URA3 gene and ARSI/CEN4 regions for maintenance of the plasmid.

Figure 2.8 pTZ18R Multifunctional Phagemid.

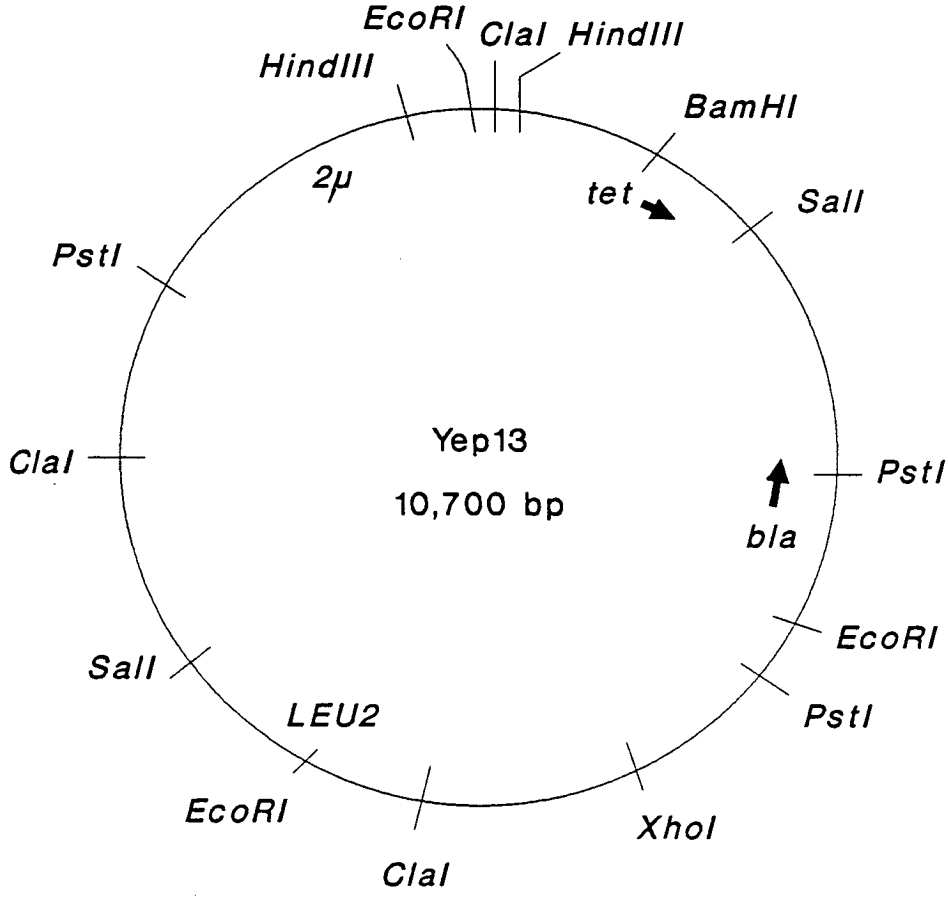


pUC18 Polylinker:

EcoRI-SacI-KpnI-SmaI-XmaI-BamHI-XbaI-SalI-AccI-HincII-PstI-SphI-HindIII

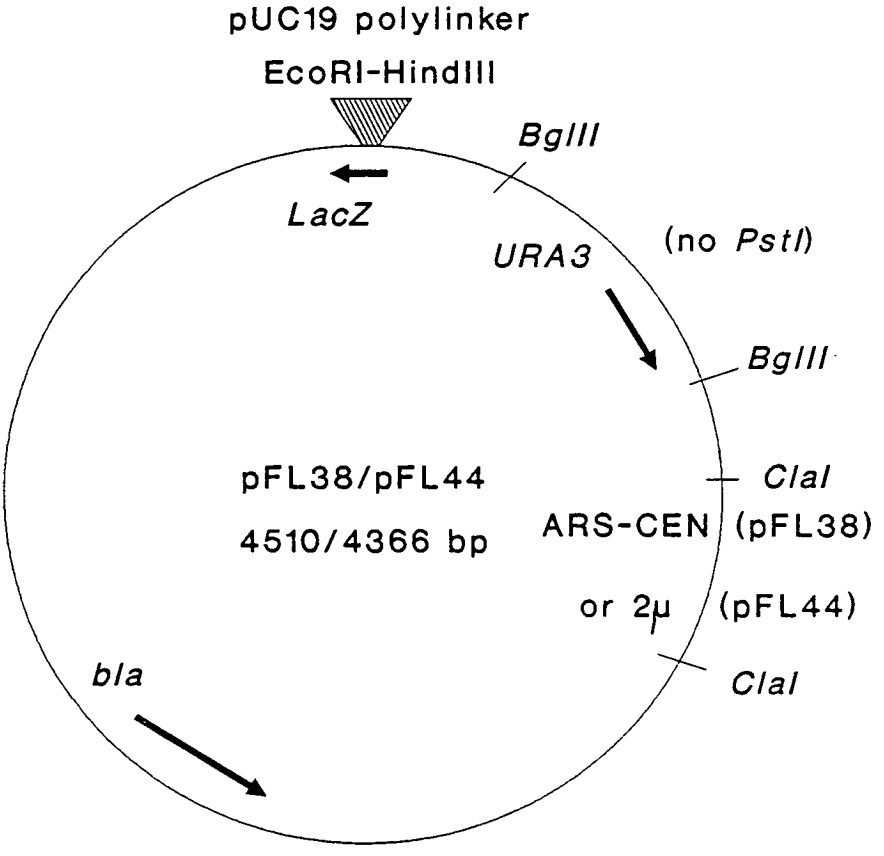
The plasmid pTZ18R contains the lacZ gene and multiple cloning site from pUC18, the T7 promoter and the site for reverse primer (Mead et al., 1986).

Figure 2.9 YEp13 Yeast Cloning Vector.



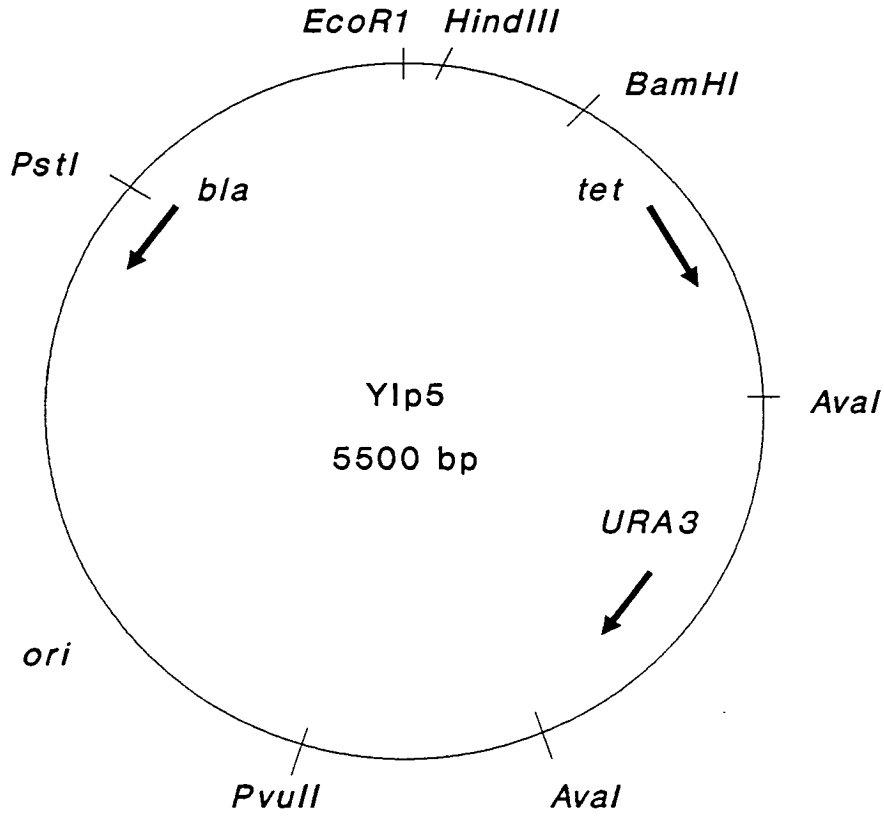
Plasmid YEp13 contains the 2μ origin of replication, and the LEU2 gene (Broach et al., 1979). Fragments can be cloned into the unique BamHI site, and recombinants screened by insertional activation of the tet gene.

Figure 2.10 pFL38 and pFL44 Yeast Cloning Vectors.



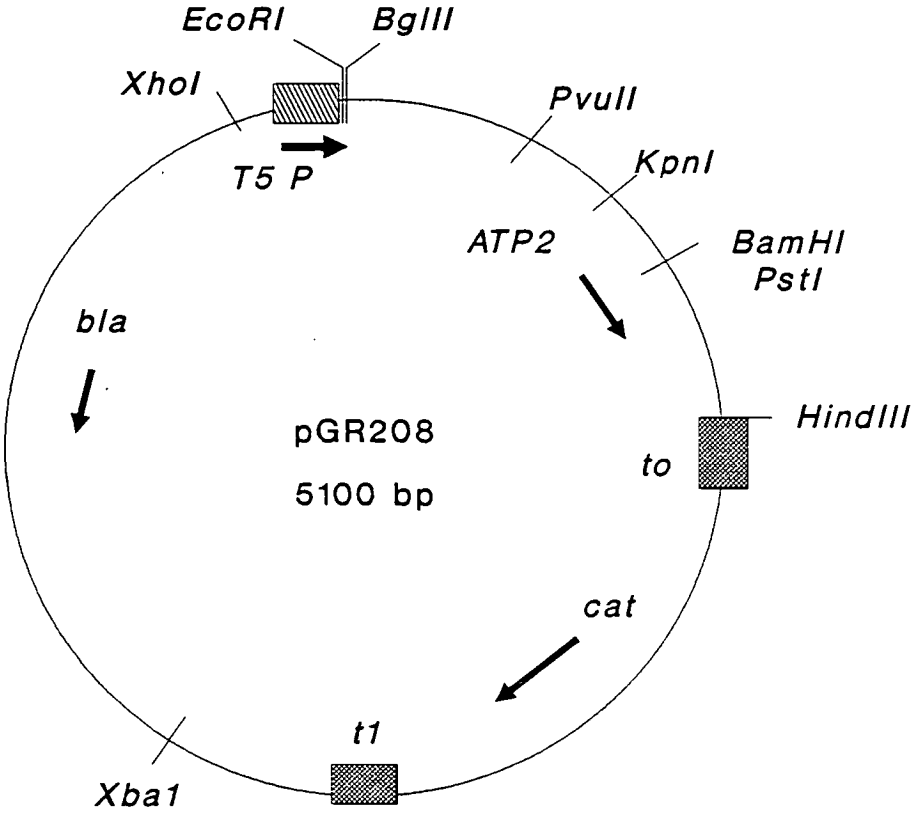
Plasmid pFL38 was constructed by F. Lacroute and is based on pUC19. It contains the URA3 gene on a 1.2 kb BglII fragment inserted at the AluI (629) site of pUC19 using BglII linkers. It also contains the centromer of chromosome 6 with an uncharacterized ARS on a 0.8 kb ClaI fragment inserted at the AluI (747) site of pUC19 using ClaI linkers. Plasmid pFL44 is identical except that it contains the 2μ origin of replication on a 0.51 kb ClaI fragment instead of the ARS-CEN region.

Figure 2.11 Ylp5



Plasmid Ylp5 contains the URA3 gene on a 1.1 kb inserted into the AvaI site of pBR322. It contains no origin of replication for yeast.

Figure 2.12 Plasmids pGR208 and pEE1



Plasmid pGR208 was obtained from Dr. G Reid, and consists ofthe ATP2 coding region originating from pJ14 (Saltzgaber-Muller et al., 1983) inserted into the cloning site of pDS6 (Figure 2.6).

Plasmid pEE1 is identical to pGR208 except that the HindIII site has been removed by filling in, and inserting a BglII site using BglII linkers.

2.3.2 Media for *S.cerevisiae*

YPD	**per litre**
Yeast Extract	10 g
Peptone	20 g
Glucose	20 g

YPG	**per litre**
Yeast Extract	10 g
Peptone	20 g
Glycerol	30 ml

SD	**per litre**
Yeast Nitrogen Base	1.7 g
$(NH_4)_2SO_4$	5 g
Glucose	20 g
Amino acids	50 mg

SDG	**per litre**
Yeast Nitrogen Base	1.7 g
$(NH_4)_2SO_4$	5 g
Glucose	1 g
Glycerol	30 ml
Amino acids	50 mg

2% Lactate	**per litre**
Glucose	1 g
Yeast Extract	3 g
KH_2PO_4	1 g
NH_4Cl	1 g
$CaCl_2.2H_2O$	0.5 g
NaCl	0.5 g
$MgSO_4.7H_2O$	0.73 g
20% Na lactate	100 ml

20% Na Lactate pH 6	
Na Lactate	106 ml
dH_2O	200 ml
pH to 6.0 with lactic acid	
make up to 400 ml with dH_2O	

Potassium Acetate Medium (for Sporulation).

	per litre
Potassium acetate	20 g
Yeast extract	2.2 g
Glucose	0.5 g

Plates were made by the addition of 20 g agar per litre to the above media.

2.4 CHEMICALS AND REAGENTS

All chemicals and reagents were of the highest quality commercially available, and were obtained from Sigma, Poole, Dorset, or BDH, Poole, Dorset, unless otherwise stated. Phenol was redistilled prior to use.

2.4.1 Radioactive Substances

All radioactive chemicals ([^{35}S]-methionine, [^{35}S]-cysteine, [^{35}S]-dATP, and α-[^{32}P]-dCTP) were obtained from Amersham International plc, Amersham, UK.

2.4.2 Enzymes

T_4 DNA Ligase, restriction endonucleases, Klenow fragment of DNA polymerase were obtained from Gibco-BRL, Paisley, UK. Pyruvate kinase was obtained from Boehringer Mannheim (BCL), Lewes, Sussex. Proteinase K (fungal) was obtained from BDH. Pancreatic ribonuclease A was obtained from Sigma, and was boiled for 10 minutes to remove any DNase activity before use. Yeast lytic enzyme (lyticase) from *Arthrobacter luteus* was obtained from ICN Biochemicals, Cleveland, Ohio. Sequenase was obtained from United Biochemical Corporation, Cleveland, Ohio. β-glucuronidase was obtained from Sigma.

2.4.3 Antisera and Reticulocyte Lysate

Antibodies against the β-subunit of the F_1 ATPase were a gift from Dr G. Schatz, University of Basel. Antisera against cytochrome b_2 was raised in rabbits by Dr G Reid. HRP-conjugated goat anti-rabbit IgG was obtained from BioRad Laboratories. Richmond, California, USA. Rabbit reticulocyte lysate was purchased from Amersham International as a nuclease-treated (messenger dependent) kit.

2.5 TRANSFORMATION PROCEDURES

2.5.1 Transformation of *E. coli*

E. coli was transformed with plasmid DNA essentially as described by Mandel and Higa (1970). *E. coli* cells were grown in 20 ml of LB at 37°C to an OD_{600} of 0.3, and were harvested by centrifugation. After resuspension in 10 ml of cold sterile 100 mM $CaCl_2$ and incubation on ice for 20 minutes or longer, cells were pelleted and resuspended in 2 ml of 100 mM $CaCl_2$.

Plasmid DNA was added to 100 µl of cold TMC (10 mM Tris.Cl pH 7.5, 10 mM $MgCl_2$, 10 mM $CaCl_2$) to give a final concentration of approximately 50 ng/ml. 200 µl of cells were added to the DNA solution, and incubated on ice for 30 minutes. Cells were heat-shocked by incubating at 42°C for 2 minutes, and allowed to phenotypically express the plasmid marker by adding 1 ml LB and incubating at 37°C for one hour. Cells were then pelleted and spread onto LB plates with selection (generally ampicillin at 50 µg/ml). The plates were incubated at 37°C overnight.

2.5.2 Transformation of *S. cerevisiae*

S. cerevisiae was transformed with plasmid and linear DNA using the lithium acetate procedure as described by Ito *et al* (1983). Yeast cells were grown to an OD_{600} of 0.4, harvested by centrifugation, washed in 10 ml TE (10 mM Tris.Cl pH7.4, 1 mM Na_2EDTA), and resuspended in 5 ml TE. 208 µl 2.5 M lithium acetate was added to give a final concentration of 0.1 M, and the cells were incubated at 30°C for one hour with gentle shaking. DNA was mixed with 100 µl distilled water to give a final concentration of 100 µg/ml (plasmid DNA) or 200 µg/ml (linear DNA), and 200 µl competent yeast cells were added. Cells were incubated at 30°C for 30 minutes, and after the addition of 700 µl polyethylene glycol 4000, the cells were incubated for a further hour at 30°C. Cells were heat-shocked by incubating at 42°C for 5 minutes, pelleted by centrifugation at 12,000 x g for 1 minute, washed twice in distilled water, and plated out on selective media. Plates were incubated at 30°C for 3-5 days.

2.6 ISOLATION OF NUCLEIC ACIDS

The following solutions were routinely used in nucleic acid techniques:

TE - 10 mM Tris.Cl pH 7.4, 1 mM Na_2EDTA

Phenol - saturated with TE.

2.6.1 Isolation of Plasmid DNA from *Escherichia coli*

Plasmid DNA was isolated from *Escherichia coli* using the alkaline lysis method essentially as described by Birnboim and Doly (1979). The following solutions were used:

TEG - 50 mM glucose, 25 mM Tris.Cl pH 8.0, 10 mM Na_2EDTA

Lysis Solution - 1% SDS, 0.2 M NaOH

2.6.1.1 Small Scale Isolation of Plasmid DNA from *E. coli*.

This method was used to give approximately 5 μg plasmid DNA which was suitable for restriction endonuclease digestion analysis, and for transforming *Saccharomyces cerevisiae* and *Escherichia coli.*

A single colony was picked and grown overnight in 5 ml LB with selection for the plasmid (generally ampicillin at 50 μg/ml). Cells were harvested by spinning at 2,000 x g for 5 minutes, and were resuspended in 100 μl TEG (Section 2.6.1) and transferred to a microcentrifuge tube. Cells were lysed by the addition of 200 μl of Lysis Solution. After 5 minutes incubation on ice, 150 μl of 3 M Na acetate pH 5.2 was added, the tubes were inverted several times, and left on ice a further 5 minutes. The precipitate was removed by centrifugation at 15,000 x g for 10 minutes, and the clear lysate was decanted off and extracted with phenol:chloroform:isoamyl alcohol (24:24:1) to denature any protein present. DNA was precipitated out of solution by the addition of 900 μl of ice-cold ethanol, and was isolated by centrifugation at 15,000 x g for 10 minutes. The pellet of DNA was dried and resuspended in 50 μl TE.

2.6.1.2 Medium Scale Isolation of Plasmid DNA from *E. coli*.

This method was used to isolate plasmid DNA of reasonable purity which was suitable for preparing fragments for subcloning, and for transforming *Saccharomyces cerevisiae* and *Escherichia coli*, and generally gave a yield of 40 μg.

A single colony was picked and grown overnight in 40 ml LB (Section 2.3.1) with appropriate selection. Cells were harvested and were resupended in 2 ml TEG (Section 2.6.1). Lysozyme was added to a concentration of 2 mg/ml, and the cells were incubated on ice for 30 minutes. Cells were lysed by the addition of 4 ml Lysis Solution. 3 ml of 3 M sodium acetate pH 5 was added, and after 30 minutes on ice, the precipitate formed was removed by centrifugation at 12,000 x g for 20 minutes. The supernatant was removed and plasmid DNA was precipitated by the addition of 16 ml cold ethanol. DNA was isolated by centrifugation at 12,000 x g for 10 minutes and was resuspended in 2 ml Low Salt Buffer (0.1 M sodium acetate, 1 mM Na_2EDTA, 0.1% SDS, 40 mM Tris Cl pH 8.0), and protein was removed by extracting with 2 ml phenol:chloroform:isoamyl alcohol (50:50:1). The phenol layer was back-extracted with 2 ml Low Salt Buffer, the aqueous phases combined and the DNA was then precipitated with 8 ml cold ethanol. After centrifugation at 12,000 x g for 10 minutes the pellet was dried and resuspended in 200 µl TE. To remove RNA, the solution was incubated at 37°C for 1 hour with 20 µl pancreatic ribonuclease (1 mg/ml). After extracting with phenol:chloroform:isoamyl alcohol, the DNA was precipitated with ethanol as described previously. The pellet of DNA was resuspended in 200 µl TE. (Section 2.6.1)

2.6.1.3 Large Scale Isolation of Plasmid DNA from *E. coli*

This method was used to isolate large quantities of pure DNA suitable for *in vitro* transcription, and yielded approximately 500 µg plasmid DNA. A single colony of *Escherichia coli* was picked and grown in 1 litre of LB with selection for the plasmid to an OD_{600} of 0.6. DNA was then amplified by adding 1.7 ml of 100 mg/ml choloramphenicol in ethanol and the culture was grown shaking overnight at 37°C. Cells were harvested by centrifugation, and resuspended in 9 ml TEG. Lysozyme was added to 2 mg/ml and after 15 minutes incubation on ice, the cells were lysed by the addition of 30 ml Lysis Solution (Section 2.6.1). The addition of 22.5 ml of 3 M sodium acetate pH 5

and incubation on ice for a further 30 minutes led to the formation of a precipitate which was removed by centrifugation at 16,000 x g for 30 minutes at 4°C. The supernatant was mixed with 45 ml isopropanol and the precipitated plasmid DNA was pelleted by centrifugation at 10,000 x g for 10 minutes. After drying, the pellet was resuspended in TE (section 2.6.1) to a final volume of 8 ml into which 7.75 g caesium chloride was dissolved. The precipitate of RNA which formed was removed by centrifugation at 9,000 x g for 30 minutes at 20°C, and the supernatant was tranferred to a polyallomer centrifuge tube containing 300 μl of 10 mg/ml ethidium bromide. The tube was centrifuged at 35,000 x g for 48 hours to allow a density gradient to reach equilibrium in the tube. Supercoiled plasmid DNA was visible as a band, and was removed from the tube using a syringe. Ethidium bromide was removed from this DNA by extracting three times with 2 ml isopropanol saturated with NaCl and water. 8 ml TE was added to the aqueous layer, and the plasmid DNA was precipitated by adding 8 ml isopropanol. After centrifugation the pellet was dried and then dissolved in 200 μl TE.

2.6.2 Isolation of Single-Stranded DNA from *E. coli.*

Single-stranded DNA from plasmids with the F1 origin of replication (phagemids) was prepared from *E. coli* using M13 helper phage. This method yielded approximately 1 μg single-stranded DNA which was suitable for use in sequencing reactions.

A single colony of a suitable *E. coli* host containing the phagemid was grown in 2 ml selective medium to mid-log phase (OD_{600} of 0.5). To this, M13K07 helper phage was added to give a mulitplicity of infection of 10, and the culture was shaken vigorously. After 1 hour, 400 μl of infected cells were mixed with 10 ml selective medium, and kanamycin was added to a final concentration of 70 μg/ml to select for the phage. The culture was grwon overnight, with vigourous shaking to give good aeration.

Cells were removed from the culture supernatant by centrifuging 1.5 ml of the overnight culture at 12,000 x g for 5 minutes. To precipitate the phage,

1.2 ml of supernant was added to 0.3 ml of NaCl/PEG Solution (2.5 M NaCl, 20% polyethylene glycol 4000), and left at room temperature for 15 minutes. The phage were pelleted by centrifugation at 12,000 x g for 5 minutes, and after removing all NaCl/PEG Solution, were resuspended in 100 µl TE (Section 2.6.1). DNA was isolated by vortexing the phage with 50 µl phenol removing the aqueous layer after centrifugation for 1 minute, and extracting with 500 µl diethyl ether. To precipitate the DNA, 10 µl of 3 M Na acetate pH 5 was added with 250 µl ethanol, and the solution left at -20°C for 1 hour. The DNA pellet was isolated by centrifugation at 12,000 x g for 5 minutes, dried and resuspended in 50 µl TE.

2.6.3 Isolation of DNA from *S. cerevisiae*

2.6.3.1 Isolation of Plasmid DNA from *S. cerevisiae.*

This method resulted in sufficient plasmid DNA to transform *Escherichia coli*, from which larger quantities could be isolated if necessary and is describe by Sherman *et al* (1979).

A single colony of *S. cerevisiae* was grown in 100 ml selective media to an OD_{600} of 0.6. Cells were harvested and resuspended in 2 ml 0.1 M Tris.SO_4 pH 9.4, 1 mM DTT, and incubated at 30°C for 15 minutes. Cells were pelleted by centrifugation and resuspended in 2.5 ml 1.2 M sorbitol, 10 mM Tris.Cl pH 7 and lyticase was added to a concentration of 40 µg/ml. Cells were incubated at 30°C and the formation of sphaeroplasts was monitored by mixing 5 µl of cells with 5 µl water on a microscope slide, and observing lysis. Sphaeroplasts were centrifuged at 2,000 x g for 5 minutes, washed twice in 1.2 M sorbitol, resuspended in 2.5 ml of 1.2 M sorbitol and lysed by the addition of 2.5 ml Lysis Solution (0.2 M NaOH, 1% SDS). After the addition of 2.5 ml of 3 M potassium acetate pH 5, a precipitate was allowed to form. This was then removed by centrifugation at 10,000 x g, the supernatant transferred to a clean tube and the plasmid DNA precipitated by the addition of 14 ml cold ethanol. After centrifugation at 10,000 x g for 10 minutes, the pellet of DNA was dried and resuspended in 400 µl TE,

extracted once with phenol:chloroform:isoamyl alcohol (24:24:1), and then with chloroform:isoamyl alcohol, before precipitating again with ethanol. The pellet was resuspended in 50 µl TE, and 5 µl was used to transform *E. coli.*

2.6.3.2 Isolation of Chromosomal DNA from *S. cerevisiae.*

A single colony of yeast was grown in 10 ml YPD to an A_{600} of 0.7. Cells were harvested, washed once in with water and resuspended in Sphaeroplasting Buffer (0.5 ml 0.9 M sorbitol, 0.05 M sodium phosphate buffer pH 7.5, 14 mM 2-mercaptoethanol) containing 40 µg/ml lyticase. Cells were incubated at 30°C for 30 minutes to allow sphaeroplasts to form, and then 50 µl of 0.5 M Na_2EDTA was added. To lyse the cells, 50 µl 10% SDS was added, with 100 µl proteinase K (5 mg/ml), and incubated at 65°C for 20 minutes. The solution was extracted with 500 µl phenol:chloroform:isoamyl aclohol (24:24:1) and the aqueous layer carefully transferred to a clean tube. 500 µl ethanol was added, and a precipitate of chromosomal DNA was allowed to form. DNA was spooled out of solution using a microcapillary tube and transferred to a clean tube. The pellet was resuspended in 500 µl TE and treated with 5 µl pancreatic ribonuclease A (2 mg/ml) for 15 minutes at 60°C. After extracting with phenol: chloroform: isoamylalcohol (24:24:1), the DNA was precipitated by the addition of 500 µl ethanol, removed as above to a clean tube, dried and resuspended in 50 µl TE.

2.6.4 Isolation of RNA from *S. cerevisiae.*

This method was used to isolate total messenger RNA (mRNA) for Northern analysis. The following solution was used:

TNE - 50 mM Tris.Cl pH 7.5, 5 mM Na_2EDTA, 100 mM NaCl, treated with diethyl pyrocarbonate (DEPC) at 0.1%, and then autoclaved to remove the DEPC.

A single colony of *S. cerevisiae* was inoculated into 200 ml of media (either YPD or SD) and grown to an OD_{600} of 0.6. Cells were chilled on ice, and then harvested by centrifugation at 4°C. The cell pellets were washed in

5 ml cold TNE and then resuspended in 1 ml cold TNE. Approximatetly 0.3 ml acid washed glass beads (40 mm 40 mesh) were added together with 200 µl 20% SDS and 4 ml phenol and the cells were broken by vortexing the contents of the tube for 1 minute at maximum speed. 3 ml of TNE was added, the tube vortexed again, and the layers separated by centrifugation. The aqueous layer was removed to a fresh tube and extracted with 4 ml phenol: chloroform: isoamyl alcohol (24:24:1), then with 4 ml of chloroform: isoamyl alcohol (50:1) and finally, RNA was precipitated with the addition of 400 µl sodium acetate and 8 ml ethanol. After 1 hour at -20°C, the precipitate of RNA was pelleted by centrifugation at 10,000 x g, washed in 66% ethanol, dried and resuspended in 100 µl distilled water (DEPC-treated). The yield was approximately 0.5 mg of RNA.

2.7 DNA MANIPULATION TECHNIQUES

2.7.1 Restriction Endonuclease Digestion

For analysis of plasmid DNA, restriction endonuclease digestion was carried out as follows. Approximately 1 µg of DNA was dissolved in 10 µl of the appropriate buffer supplied (Gibco-BRL). One unit of restriction endonuclease was added, and the DNA incubated at the appropriate temperature (generally at 37°C for one hour). Plasmid fragments were separated by agarose gel electrophoresis (Section 2.9.1).

For preparation of fragments of DNA for subcloning, approximately 10 µg of plasmid DNA was dissolved in 50 µl of the appropriate buffer supplied. 5 units of the restriction endonuclease were added, and the reaction carried out as described above.

2.7.2 Filling in of Protruding 5' ends of Fragments

Protruding 5'ends were filled in as described by Wartell and Reznikoff (1980) using the DNA polymerase activity of the large subunit (Klenow fragment) of DNA polymerase I from *E. coli*. 1 µg of the fragment of DNA in 21.5 µl of distilled water was added to 2.5 µl of 10 x Nick Translation Buffer

(0.5 M Tris.Cl pH 7.5, 0.1 M $MgSO_4$, 10 mM DTT, 500 μg/ml BSA) and 1 μl of a 2 mM solution of all 4 dNTPs. 2 units of the Klenow fragment of DNA polymerase I was added and was incubated at 22°C for 30 minutes. The enzyme was inactivated by heating to 70°C for 5 minutes.

2.7.3 Ligation of DNA ends

The ligation of DNA ends was used in the subcloning of isolated fragments of DNA into other plasmid vectors and also to self-ligate vector DNA after the removal of stretches of nucleotides. Ligations were carried out as described by Maniatis (1979) using T_4 ligase. The amount of DNA used varied depending on whether the ends to be ligated were cohesive or blunt, and whether the subcloning was directed or not. Generally, for subcloning, vector DNA was present at a concentration of 50 ng/ml, and was mixed with the fragment at a ratio of approximately 1:3. The reaction was carried out in Ligation Buffer (50 mM Tris.Cl pH 8.0, 10 mM $MgCl_2$, 10 mM DTT, 1 mM spermidine, 1 mM ATP pH 7.4, 100 μg/ml BSA). T_4 DNA ligase was added to a give a final concentration of 10 units/ml, and the reaction carried out at 16°C overnight.

2.8 DNA SEQUENCING

2.8.1 Sequencing of Plasmid DNA.

Plasmid sequencing was carried out to verify the nucleotide sequence of certain constructs achieved through DNA manipulations. The dideoxy chain termination method used was essentially that described by Sanger *et al* (1977) as modified by Wallace *et al* (1981) specifically for sequencing double stranded plasmid DNA. The DNA used for sequencing was prepared either by the Medium Scale method (Section 2.6.1.2) or Large Scale method (2.6.1.3).

Annealing of Template and Primer

2 μg of plasmid DNA was precipitated with ethanol, dried down and resuspended in 10 μl sequencing buffer (60 mM NaCl, 50 mM Tris.Cl pH 7.5, 7 mM magnesium acetate, 7 mM dithiothreitol). Approximately 6 pmoles of a suitable oligonucleotide primer in 3 μl of distiled water was added, and the mixture boiled for 3 minutes in a microcentrifuge tube. The contents of the tube were subjected to a brief centrifugation, and incubated at 37°C for 20 minutes.

Sequencing Reactions

Termination mixes were made up as follows:

	μl added			
Nucleotide	**T**	**C**	**G**	**A**
0.5 mM dTTP	2.5	50	50	35
0.5 mM dCTP	50	2.5	50	35
0.5 mM dGTP	50	50	2.5	35
3.3 mM ddTTP	102.5	0	0	0
0.1 mM ddCTP	0	102.5	0	0
0.2 mM ddGTP	0	0	102.5	0
0.13 mM ddATP	0	0	0	102.5
10mM Tris.Cl pH 8				87.5

The annealed template/primer mixture was divided in 2 μl aliquots in each of four tubes. Onto the side of each tube, 2 μl of one of termination mixes (T,C,G or A) was added. 1 unit of the Klenow Fragment of DNA polymerase I was mixed with 4 μCi of [^{35}S]-αdATP (300 Ci/mmol) in a volume of 9 μl of 10 mM Tris.Cl pH 8, and 2 μl of this mix was added to each of the 4 tubes. The reaction was started by centrifuging the tubes briefly, and was carried out at 30°C for 15 minutes. 2 μl of Chase Mix (0.5 mM dATP,0.5 mM dCTP, 0.5 mM dTTP, 0,5 mM dGTP) was added and the mixture incubated for a further 20 minutes.

The samples were denatured by the addition of 4 μl formamide-dyes mix

before electrophoresing on an urea polyacrylamide gel (Section 2.9.4).

Formamide Dyes Mix

100 ml formamide (deionized for 30 minutes with Amberlite MB1)

30 mg xylene cyanol

30 mg bromophenol blue

4 ml 0.5M Na Na_2EDTA

2.8.2 Sequencing of Single-Stranded DNA Using Sequenase

DNA sequencing using the "Sequenase" enzyme (a derivative of T7 DNA polymerase described by Tabor and Richardson, 1987) was carried out using a kit supplied by United States Biochemical Corporation, Cleveland, Ohio. The procedure used is described by UBSC in their booklet Sequencing with Sequenase Version 2 (Third Edition). An increase in labelling of short chains near the priming site was achieved by adding 1 μl Manganese Buffer (supplied in the kit) to the labelling reaction.

2.9 GEL ELECTROPHORESIS OF NUCLEIC ACIDS

2.9.1 Agarose Gel Electrophoresis

Agarose gels were used to separate fragments resulting from the restriction endonuclease digestion of plasmid DNA (Section 2.7.1). Also restriction digestions of chromosomal DNA were separated on agarose gels prior to carrying out Southern Transfer (Section 2.10.1). A crude analysis of RNA from *in vitro* transcription was also achieved using this method (Section 2.17). The following buffers were used:

10 x TBE	**per litre**	**Loading Buffer**
Tris base	108g	0.25% bromophenol blue
Boric acid	55g	15% Ficoll 400
0.5M Na_2EDTA pH 8.0	40ml	

In general, fragments in the size range 0.5 kb to 10 kb were separated on an 0.8% agarose gel which was prepared by melting the agarose in 1 x TBE

and allowing to set in a gel cast. Approximately 1 μg of DNA was mixed with loading buffer, and loaded into the wells of the gel which was submerged in 1 x TBE in an electrophoresis tank. Electrophoresis was carried out at a current of 100 mA. The gel was stained using ethidium bromide, and DNA was visualized using ultraviolet light at 280 nm. Where it was necessary to separate smaller fragments, a higher concentration of agarose was used.

2.9.2 Electroelution of DNA Fragments

In order to isolate fragments of DNA for subcloning, the DNA was digested with the appropriate restriction endonuclease (Section 2.7.1), and the fragments produced were electrophoresed on an agarose gel as described above. The fragment required was excised from the gel using a sharp blade, and the DNA eluted from the agarose using a Biotrap (Schleicher and Schuell) according to the manufacturers instructions. Electroelution generally took 1 hour at a voltage of 100 V. The DNA was recovered by reversing the current for 5 seconds, and then removing the 200 μl of buffer between the membranes. The DNA was precipitated out of the buffer by adding 20 μl of sodium chloride and 400 μl of ethanol, and then pelleting by centrifugation. The DNA was resuspended in TE (10 mM Tris.Cl pH 7.4, 1 mM Na_2EDTA).

2.9.3 Agarose Gels for the Separation of RNA

RNA isolated from *S. cerevisiae* was separated on agarose gels prior to Northern blotting (2.10.1.2) using the method of McMaster and Carmichael (1977). The following solutions were used:

Glyoxal Mix
100 μl glyoxal (30%) deionized to pH 6
250 μl formamide pH 7
10 μl 0.5 M sodium phosphate
40 μl distilled water

A 1.5% agarose gel was prepared by mixing 0.75 g of agarose in 49 ml distilled water and 50 µl diethyl pyrocarbonate for 30 minutes. The agarose was melted by boiling and, when cool, 1 ml of 0.5 M sodium phosphate was added, and the gel cast. The RNA sample was prepared by mixing 5 µl of RNA with 20 µl of glyoxal mix and incubating at 50°C for 30 minutes. The sample was loaded into the wells of the gel, which was surrounded by 0.01 M sodium phosphate buffer pH 7, and the RNA electrophoresed into the gel before adding more buffer to submerge the gel. Electrophoresis was carried out at 200 mA for 2 hours, recirculating the buffer every 10 minutes.

2.9.4 Sequencing Gels

Urea-acrylamide gels were prepared for the separation of nucleotides after sequencing reactions (Section 2.8).

Sequencing Gel Solution	**per litre**
Acrylamide	57 g
Bisacrylamide	3 g
Urea	420 g
dH_2O	to 900 ml

The solution was deionized by stirring with deionizing resin and filtered through Whatman No.1 paper.

Sequencing gels were made by mixing 54 ml Sequencing Gel Solution with 6 ml 10 x TBE (Section 2.9.1), and adding 120 µl of TEMED and 120 µl of 10% ammonium persulphate before pouring between sequencing plates, and allowing to polymerize. Gels were electrophoresed in 1 x TBE at 65 Watts.

2.10 NUCLEIC ACID HYBRIDIZATION TECHNIQUES

In order to detect specific sequences in either RNA or chromosomal DNA from the cell, nucleic acid was separated on an agarose gel and transferred to a nylon membrane before hybridization with a DNA probe.

2.10.1 Transfer of Nucleic Acids to Filters

The capillary blotting method used for transfer was essentially that described by Southern (1975), and as detailed below for the transfer of chromosomal DNA (Southern Transfer) or RNA (Northern Transfer).

2.10.1.1 Southern Transfer

Chromosomal DNA was prepared as described in Section 2.6.3.2, digested with a suitable restriction endonuclease, and fragments were separated on an 0.8 % agarose gel by electrophoresing in 1 x TBE (Section 2.9.1). The gel was placed in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes, and then in neutralizing solution (1.5 M NaCl, 0.5 M Tris.Cl pH 7.2, 1 mM Na_2EDTA) for 30 minutes. The gel was place upside down on a piece of 3MM filter paper, the ends of which were resting in reservoir of 20 x SSC (3 M NaCl, 0.3 M Na_3 citrate). A sheet of nylon membrane (Hybond-N, Amersham) was cut to the size of the gel and placed on top and air bubbles were eliminated. Three layers of filter paper, presoaked in 2 x SSC were placed on top of the membrane followed by a 10 cm stack of absorbant paper towels, and finally a glass plate and a weight to ensure even transfer. Transfer was allowed to proceed overnight, after which the filter was removed, washed in 2 x SSC and allowed to dry. The DNA was crosslinked to the nylon membrane by wrapping in saran wrap and placing upside down on a UV transilluminator for 5 minutes.

2.10.1.2 Northern Transfer

Total RNA was prepared as described in Section 2.6.4 and was then electrophoresed on a denaturing agarose gel as described in Section 2.9.3. Transfer of RNA to the nylon membrane was essentially as described for Southern Transfer above, using the method described by Thomas (1980) except that the membrane was baked for 2 hours at $80^{o}C$ after transfer to reverse glyoxalation and to therefore allow the subsequent hybridization steps to take place efficiently.

2.10.2 Detection Using Radioactive Probes.

2.10.2.1 Radioactive Probe Synthesis.

The methods used for labelling fragments of DNA to be used as probes involved the priming of random hexanucleotides to the denatured probe (Feinberg and Vogelstein, 1983), followed by DNA synthesis in the presence of [^{32}P]-αdCTP.

The following solutions were used:

Nucleotide Stocks (dATP, dGTP, dTTP) Each at a concentration of 0.1 M in 3 mM TrisCl pH 7, 0.2 mM Na_2EDTA

Solution O - 1.25 M Tris.Cl pH 8, 125 mM $MgCl_2$

Solution A - 1 ml Solution O, 18 μl 2-mercaptoethanol, 5 μl each of dATP, dGTP, dTTP.

Solution B - 2 M Hepes pH 6.6.

Solution C - Hexadeoxyribonucleotides in TE at 90 OD units/ml.

OLB - Solutions A:B:C in a ratio of 10:25:15

Approximately 50 ng of the DNA fragment to be used as a probe was precipitated with ethanol, dried and resuspended in 32.5 μl of distilled water. The DNA was boiled for 5 minutes, chilled on ice, and 10 μl of OLB was added together with 2 μl BSA (10 mg/ml). The DNA and hexanucleotides were allowed to anneal by incubating at 37^{o}C for 10 minutes. Then, 5 μl of [^{32}P]-adCTP was added together with 2 units of Klenow fragment of DNA polymerase, and polymerization allowed to procede at room temperature for 5 hours or more. The reaction was stopped by boiling for 10 minutes, and the denatured probe was used for hybridization to membrane-bound nucleic acid (Section 2.10.3).

2.10.2.2 Hybridization Conditions for Radioactive Probes

The following solutions were used for both RNA and DNA hybridizations

using radioactive probes:

Prehybridization Solution		**100 x Denhardt's**
20 x SSC	7.5 ml	2% BSA
100 x Denhardt's	1.25 ml	2% Ficoll
10% SDS	1.25 ml	2% polyvinylpyrollidone
dH_2O	15.0 ml	
Total	25.0 ml	

The nylon membrane to which the RNA or DNA was bound was placed in a bag with the prehybridization solution, and denatured salmon sperm DNA was added at a concentration of 0.1 mg/ml. Pre-hybridization was carried out at 65°C for 3 hours with shaking. For hybridization, 50 ng of denatured labelled probe was added to the bag and hybridization was carried out at 65°C for 12 hours with shaking, before washing. Membranes were incubated with increasingly stringent solutions to remove unbound probe and to reduce non-specific binding as follows:

Twice with 50 ml of 2 x SSC at 65°C for 15 minutes;

Once with 50 ml of 2 x SSC, 0.1% SDS at 65°C for 30 minutes;

Once with 50 ml of 0.1 x SSC at 65°C for 10 minutes;

Membranes were allowed to dry and were autoradiographed at -70°C

2.10.3 Nonradioactive Detection.

2.10.3.1 Synthesis of Digoxigenin-labelled DNA Probes

Random primed DNA labelling with digoxigenin-dUTP was carried out using a kit supplied by Boehringer Mannheim (BCL) according to the maunfacturers instructions. The following solutions were used:

Hexanucleotide Mixture - Hexadeoxyribonucleotides at 90 OD units/ml

dNTP Labelling Mixture - 1 mM dCTP, 1 mM dGTP, 1 mM dATP, 0.65 mM dTTP, 0.35 mM digoxigenin-dUTP, pH 6.5

Approximately 50 ng of DNA to be used as a probe was precipitated with ethanol, dried and resuspended in 15 µl of distilled water. The DNA was

denatured by boiling for 10 minutes, and 2 µl of hexanucleotide mix was added together with 2 µl of dNTP Labelling mix and 2 units of Klenow fragment. Probe synthesis was allowed to procede for at least an hour and the reaction was stopped by the addition of 1 µl of 0.2 M Na_2EDTA. Approximately 10 µl of this solution was used for hybridization after denaturation by boiling.

2.10.3.2 Hybridization and Immune-detection of Diogoxigenin Labelled Probes.

The detection of digoxigenin-labelled probes was carried out according to the instruction supplied by Boehringer Mannheim (BCL) in their Nonradioactive labelling kit. The following solutions were used:

Hybridization Solution - 5 x SSC, 0.5% (w/v) Blocking Reagent (BCL), 0.02% SDS

TN - 100 mM Tris.Cl pH 7.5, 150 mM NaCl

Developing Buffer - 100 mM Tris.Cl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$.

Colour Solution -

45 µl nitroblue tetrazolium salt solution (75 mg/ml in 70% dimethylformamide) and 35 µl 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt solution (50 mg/ml in dimethyl formamide) in 10 ml Developing Buffer.

Stop Solution - 10 mM Tris.Cl pH 8, 1 mM EDTA.

The membrane was prehybridized in 20 ml Hybridization Buffer at 68°C for one hour. The solution was replaced with 20 ml hybridization solution containing 25 ng freshly denatured digoxigenin-labelled DNA, and the membrane incubated for 6 hours at 68°C with occasional shaking. The membrane was then washed twice for 5 minutes at room temperature with 2 x SSC, 0.1% SDS, and twice for 15 minutes at 68°C with 0.1 x SSC, 0.1% SDS. The membrane was washed in TN for 1 minute and then incubated for 30 minutes in Blocking Solution (0.5% Blocking Reagent in TN). After rinsing in TN, the membrane was incubated for 30 minutes with alkaline phosphatase-conjugated sheep antidigoxigenin Fab-fragments diluted

1:5000 in TN. The membrane was washed twice in TN for 15 minutes, and then washed in Developing Buffer for 1 minute, and 10 ml Colour Solution was added. The reaction was allowed to proceed in the dark until bands were visible, and was stopped by washing for 5 minutes in Stop Solution.

2.11 EXTRACTION OF TOTAL CELL PROTEIN FROM *S. cerevisiae*

Total cell protein suitable for SDS-PAGE (Section 2.12) was isolated from yeast using trichloroacetic acid (TCA) and glass beads to break the cells.

The following solution was used (Laemmli, 1970):

2 x LSB (Laemmli Sample Buffer)

1 M Tris.Cl pH 6.8	3.13 ml
SDS	2 g
Glycerol	9 ml
2-mercaptoethanol	5 ml
1% bromophenol blue	1 ml
Distilled water	32 ml

A single colony of yeast was grown in appropriate media to an A_{600} of 0.7. Cells were harvested by centrifugation, washed in distilled water and resuspended in 1 ml ice-cold 10% TCA. Approximately 2 ml of glass beads (0.45 - 0.5, 40 mesh) were added to give a thick slurry which was then vortexed for 2 minutes at full speed. After the addition of 1 ml of 10% TCA, the cells were vortexed again, and the lysate was removed to a fresh tube. The beads were washed twice with 10% TCA and washings were combined. The protein precipitate was pelleted by centrifugation at 12,000 x g for 5 minutes, and was washed twice in cold ethanol to remove the residual TCA. The protein pellet was dried and resuspended in 200 μl of 2 x LSB prior to SDS-PAGE (Section 2.12).

Protein concentration in these samples was estimated by spotting 2 μl dilutions of the cell extract on a piece of filter paper alongside 2 μl standards of bovine serum albumen (1 μg/ml to 1 mg/ml) and staining with PAGE blue 83 electran solution made up in 20% methanol, 5% acetic acid. The intensity

of the blue spot was compared to the known standards, and gave an indication of the amount of protein present.

2.12 GEL ELECTROPHORESIS OF PROTEINS

One dimensional electrophoresis on SDS-acrylamide gels was used to separate proteins according to their molecular weight, essentially as described by Laemmli (1970). The following solutions were used:

4 x Resolving Buffer

Tris base	181.6 g
SDS	4.0 g
dH_2O	600. ml
conc HCl	10.0 ml
pH to 8.8	
Made up to 1 litre with dH_2O	

4 x Stacking Buffer

Tris base	30.28 g
SDS	2.0 g
dH_2O	450.0 ml
conc HCl	15.0 ml
pH to 6.8	
Made up to 500 ml with dH_2O	

10 x Running Buffer

Tris base	30 g
Glycine	144 g
SDS	10 g
dH_2O to 1 litre	

Typically a 10% acrylamide gel was made as follows:

Resolving Gel

Acrylamide (30%:0.8% bisacrlyamide)	10.0 ml
4 x Resolving Buffer	7.5 ml
dH_2O	12.3 ml
TEMED	30 µl
Ammonium persulfate (10%)	190 µl

The gel was poured between two glass plates, overlayed with water-saturated butanol, and allowed to polymerize. A stacking gel was prepared as follows and poured on top :

Stacking Gel

Acrylamide (30%;0.8% bisacrylamide)	1.6 ml
4 x Stacking Buffer	2.5 ml
dH_2O	5.86 ml
TEMED	10 µl
Ammonium persulphate	30 µl

Samples in 2 x LSB (Section 2.11) were loaded into the wells, and gels were run at 10 volts per cm for 3.5 - 4 hours. Protein gels were stained with 1% PAGE Blue 83 electran in 20% (v/v) methanol, 5% (v/v) acetic acid.

2.13 WESTERN BLOTTING

In order to detect the presence of specific proteins in a cell extract, the proteins were electrophoresed on an acrylamide gel, transferred to a nylon membrane, and probed with an appropriate antibody probe.

2.13.1 Electrophoretic Transfer of Protein to Membranes

The electrophoretic method used for transferring proteins from acrylamide gels to nylon membranes was essentially that described by Gershoni and Palade (1983). The following buffer was used :

10 x Transfer Buffer - 250 mM Tris.Cl pH 8.3, 1.5 M glycine

After electrophoresis of the proteins (Section 2.12), the gel was soaked in Transfer Buffer for 2 minutes, and was then assembled into a "sandwich" with the gel adjacent to a piece of nylon membrane (Hybond N, Amersham), between two layers of filter paper (presoaked in 1 x Transfer Buffer) and two sponges. The proteins were electrophoresed from the gel onto the membrane by immersing the sandwich into a tank containing Transfer Buffer, and passing through a current of 1 Amp for 1.5 hours. The membrane was either dried or used immediately for immunodetection.

2.13.2 Immunodetection of Proteins

The following solutions were used:

TBS (Tris-buffered Saline) - 10 mM TrisCl pH7.5, 150 mM NaCl.

Developing Solution:-

Diansidine (5 mg/ml)	0.5 ml
0.1 M Imidazole pH 7.4	1.0 ml
30% H_2O_2	0.1 ml
dH_2O	8.4 ml

After transfer of proteins, the nylon membrane was placed in 100 ml of 20% (w/v) milk powder solution made up in TBS and incubated overnight at 25°C to block the unbound sites on the membrane. The solution was replaced with 20 ml of 5% milk powder solution made up in TBS, and 30 µl of antibody was added. After incubating for 5 hours at 25°C, the solution was discarded, and the membrane was washed in 100 ml of TBS, 4 times for 10 minutes. The membrane was placed in fresh 5% milk powder solution (as above) and 10 µl of horse radish peroxidase-conjugated anti-rabbit IgG (BioRad) was added. After 1 hour incubation, the membrane was washed as previous, and the bound peroxidase was developed by shaking with 10 ml of Developing Solution until a brown band was visible. The reaction was stopped by rinsing the membrane thoroughly in distilled water, photographing whilst wet, and leaving to dry on filter paper. Developed membranes were stored in the dark to prevent fading.

2.14 *IN VITRO* TRANSCRIPTION OF PLASMID DNA

Messenger RNA was transcribed from plasmid DNA by using *E. coli* RNA polymerase to initiate transcription from the phage T5 promoter in pDS derived constructs (Stueber *et al.,* 1984).

The following solutions were used:

5 x Transcription Buffer (TB)	**per ml**	**Final Concentration**
1 M HEPES KOH pH 7.9	100 µl	0.1 M
1 M Mg acetate	50 µl	50 mM
5 M K acetate	200 µl	1 M
1 M dithiothreitol	25 µl	25 mM
50 m M spermidine	20 µl	1 mM
0.1 M GTP	25 µl	2.5 mM
0.1 M CTP	25 µl	2.5 mM
0.1 M UTP	25 µl	2.5 mM
dH_2O (DEPC-treated)	530 µl	

Plasmid DNA was isolated on caesium chloride density gradients as described in Section 2.6.1.3. Approximately 6.3 µg of DNA was precipitated with ethanol, dried and resuspended in 6.3 µl DEPC-treated distilled water. The transcription reaction was carrried out by adding the following solutions together :

DNA (1 µg/µl)	6.5 µl
1 mM 7mGpppA cap	1.0 µl
5 x TB	2.0 µl
RNA polymerase	0.2 µl
RNasinhibitor	0.5 µl

The reaction was carried out at 37°C for 5 minutes, and then 0.5 µl of 20 mM ATP was added, and the tube incubated for a further 10 minutes at 37°C. The reaction was stopped by chilling on ice. Efficiency of transcription was assayed by electrophoresing a 2 µl aliquot on an 0.8% agarose gel. (Section 2.9.1)

2.15 *IN VITRO* TRANSLATION OF mRNA.

In vitro synthesized mRNA (Section 2.14) was translated using rabbit reticulocyte lysate obtained from Amersham. Radioactive proteins were made

by including [^{35}S]-methionine in the translation mix as follows:

Reticulocyte Lysate	16 µl
[^{35}S]-methionine (300Ci/mmol)	2 µl
mRNA (from transcription - Section 2.15)	4 µl

The mixture was incubated at 30°C for 1 hour. Radioactive protein products were analyzed using SDS-PAGE (Section 2.12) and fluorography.

2.16 ISOLATION OF YEAST MITOCHONDRIA

Mitochondria were isolated from yeast cells essentially as described by (Gasser *et al.*, 1982a). A single colony of *S. cerevisiae* was picked and grown in 2% lactate media to stationary phase. 1 litre of lactate media was inoculated with 500 µl of the starter culture and grown overnight with vigorous shaking at 30°C. Cells were harvested, washed once in distilled water and resuspended in 5 ml 0.1 M Tris.SO_4 pH 9.4, 10 mM dithiothreitol. After incubating at 30°C for 10 minutes, the cells were pelleted, and washed once in 1.2 M sorbitol, 20 mM Tris.Cl pH 7.4. After resuspending in 5 ml 1.2 M sorbitol, 20 mM Tris.Cl pH 7.4, yeast lytic enzyme was added to give a final concentration of 40 µg/ml. Cells were incubated for 15 to 30 minutes, until sphaeroplasts were formed (this was verified by mixing 5 µl of cells with 5 µl water on a microscope slide and observing lysis). The sphaeroplasts were pelleted by centrifugation at 1,000 x g for 5 minutes, and were washed twice in 1.2 M sorbitol, 20 mM Tris.Cl pH 7.4 by resuspending gently using a wide bore pipette. Sphaeroplasts were resuspended in 2 ml ice-cold Breaking Buffer (0.6 M mannitol, 20 mM HEPES KOH pH 7.4) with 1 mM PMSF, and to ensure complete lysis, the sphaeroplasts were homogenized on ice using a Dounce homogenizer. Cell debris was removed by centrifugation at 2,000 x g for 5 minutes at 4°C, the supernatant was retained on ice, and the cell pellet was resuspended in 2 ml Breaking Buffer, and homogenized again. After removing the cell debris by centrifugation, the supernatants were combined and mitochondria were pelleted by centrifugation at 9,000 x g for 10 minutes at 4°C. The post-mitochondrial supernatant was retained for subcellular

fractionation experiments. The mitochondria were resuspended in Breaking Buffer, centrifuged again at 3,500 x g to remove more cell debris, and the mitochondria were pelleted again at 9,000 x g. After washing twice in Breaking Buffer, the mitochondria were resuspended in 500 µl Breaking Buffer without PMSF. The amount of mitochondrial protein isolated was estimated by measuring the absorbance of 10 µl in 0.6% SDS at 280 nm. (An A_{280} of 0.2 approximates to 10 mg/ml protein).

2.17 *IN VITRO* IMPORT OF RADIOLABELLED PRECURSOR PROTEINS TO ISOLATED YEAST MITOCHONDRIA

This procedure was carried out essentially as described by Gasser *et al* (1982a). Radiolabelled precursor protein was prepared by transcription of the relevant plasmid DNA (Section 2.14) and translation of the message in the presence of [^{35}S]-methionine as described in Section 2.15. Yeast mitochondria were prepared as described in Section 2.16 and were kept on ice prior to carrying out the import assay as soon as possible after isolation.

IMPORT ASSAY	**µl per reaction**	**Final Concentration**
Isolated Mitochondria	50	700 µg/ml
Translation mix	16.5	
1 M KCl	12	40 mM
100 mM ATP	3	1 mM
200 mM $MgCl_2$	1.5	1 mM
20 mM K.PEP pH 7.4	75	5 mM
6 units pyruvate kinase (2U/µl)	3	20 U/ml
200 mM DTT	1.5	1 mM
1 M HEPES pH 7.4		20mM
1.5 M Mannitol		0.6M
TOTAL	300	

The assay was carried out at 28°C for 30 minutes. To stop the assay the tube was placed on ice, the contents mixed gently and then divided into 3

tubes, 100 µl into each. To quantitate the amount of precursor imported into the mitochondria and therefore protected against externally added protease, Proteinase K was added to one tube to a final concentration of 10 µg/ml; and to show that protection from proteolysis was due to a detergent soluble membrane, Proteinase K (10 µg/ml) and Triton-X100 (to 0.3%) was added to the second tube. No additions were made to the third tube, and all three tubes were incubated on ice for 15 minutes. Mitochondria were then reisolated by centrifugation at 13,000 x g for 10 minutes at 4°C. The supernatant from each tube was kept and the mitochondrial pellet was washed by gently resuspending in 100 µl 0.6 M mannitol, 20 mM HEPES pH 7.4 and reisolating by centrifugation as before. The pellets and supernatants were boiled in 1 x Laemmli Sample Buffer (Section 2.12) for 5 minutes, and the protein products were separated by electrophoresis on an SDS-PAGE gel (Section 2.13). The gel was fixed by boiling in 5% TCA for 2 minutes, and the radioactive proteins detected by fluorography by soaking the gel in 1 M sodium salycilate for 30 minutes, drying for 2 hours on a vacuum dryer, and exposing to X-ray film overnight at -70°C.

2.18 YEAST GENETIC TECHNIQUES

2.18.1 Isolation of ρ- Mutants of S. cerevisiae.

This method was used to isolate yeast which had defective mitochondrial DNA, caused by either mutation or loss. In both cases the phenotype resulted in the inability to grow on non-fermantable carbon sources such as glycerol. The method is taken from the Cold Spring Harbour Manual on Yeast Genetics (Sherman *et al.*, 1982).

A colony of the strain from which a ρ^- mutant was to be isolated was inoculated into 5 ml of SD medium (Section 2.3.2) containing 10 µg/ml ethidium bromide and nutritional requirements in a McCartney bottle to give approximately 2 x 10^6 cells per ml. The bottle was wrapped in aluminium foil to keep out light and grown overnight with shaking at 30°C. The culture was then streaked onto SDG plates (Section 2.3.2) for single colonies and

incubated at 30°C for three days. Colonies which were ρ^- were distinguished by their small size and were picked onto fresh SDG plates to confirm their phenotype.

2.18.2 Mutagenesis of *S. cerevisiae.*

The mutagen used to obtain mutants of *S. cerevisiae* was ultraviolet (UV) light at a wavelength of 260 nm. The lamp was calibrated to give a dose of 10 ergs/mm^2/second. Cells to be mutagenized were grown to stationary phase in SD medium (Section 2.3.2). 20 ml of cells were then spun down and resuspended in 20ml of sterile dH_2O. A UV survival curve was prepared by placing the cells on a glass petri dish and subjecting to UV light and taking 100 µl samples at time points from 30 seconds to 5 minutes. Serial dilutions were prepared of each time sample before plating out, and the plates incubated in the dark for 2 days before counting the number of colonies. The dose which gave approximately 50% survival was estimated from the survival curve and this was then used as the mutagenizing dose. For the mutagenesis, cells were grown to stationary phase and subjected to UV light as previously. After mutagenesis, cells were kept in the dark for all subsequent steps, and were transferred to a foil-wrapped bottle before spinning down and resuspending in 20 ml of SD medium. The pool of mutagenized cells was dispensed in 2 ml aliquots into 10 bijoux bottles and grown in the dark for 3 days. The cells were then screened for the relevant phenotype and the number of viable cells counted by plating serial dilutions on YPD plates.

2.18.3 Mating, Sporulation and Tetrad Analysis.

Diploid yeast were achieved by mating *MAT*α with *MAT*a haploid strains of *S. cerevisiae.* 20 µl sterile distilled water was spotted onto a YPD plate and a single colony of each strain was mixed in the water on the plate. After incubating at the appropriate temperature for 3 days, cells were scraped off using a toothpick onto a suitable plate to select for diploids. Diploid cells were induced to sporulate by scraping onto a potassium acetate plate (Section 2.3.2) and incubating at the appropriate temperature for 3-5 days. The

formation of ascospores was monitored by observing tetrads under the microscope. To increase sporulation in some strains, diploid cells were grown to mid-exponential phase in YPD, spun down and spotted onto potassium acetate plates.

Tetrad anaylsis was carried out after removing the walls of the asci by incubating the sporulating cells with β-glucuronidase (Sigma) for 30 minutes, and asci were dissected using a Singer micromanipulator onto YPD plates. After germination at the appropriate temperature, the progeny were tested for their relevant phenotypes by resuspending the colony in 100 μl distilled water, and spotting 10 μl onto appropriate plates.

Random spore analysis was carried out by treating the spore/diploid mixture with diethyl ether to selectively destroy diploid cells. After removing the ether phase, the mixture was plated out on non-selective plates, and spores allowed to germinate. Any surviving diploids were distinguished from haploid progeny by their larger colony size and the use of genetic markers. Haploids were then checked for their relevant phenotypes by spotting on appropriate plates.

2.19 ATPASE ASSAY.

The ability of mitochondrial ATPase to hydrolyze ATP to ADP and inorganic phosphate was assayed by measuring the amount of inorganic phophate released, and is described by Todd *et al.* (1979). The following solutions were used:

Stock B Solution - 250 mM Tris.SO_4 pH7.4, 20 mM ATP pH7.4, 20 mM $MgSO_4$, 25 mM phosphoenolpyruvate.

Pyruvate Kinase Solution - made up to 0.8 mg/ml in 10 mM Tris.SO_4 pH7.4

2.5% Ammonium Molybdenate Solution - 2.5 g ammonium molybdenate in 100 ml 5 N H_2SO_4.

Aminonaphthol Sulphonic Acid Solution - 0.5 g 1-amino-2-naphthol-4-sulphonic acid, 29 g $NaHSO_3$, 1 g Na_2SO_3, in 200 ml dH_2O.

ASSAY

The assay was carried out on isolated mitochondria, osmotically lysed and diluted as appropriate in dH_2O as follows:

Stock B Solution	20 µl
Pyruvate kinase solution	4 µl
Enzyme in dH_2O	76 µl
(dH_2O only as blank)	

After 10 minutes at 37°C, the reaction was stopped by the addition of 10 µl of 50% trichloroacetic acid. After centrifugation at 12,000 x g, 100 µl was assayed for inorganic phosphate as follows:

Sample supernatant	100 µl
dH_20	650 µl
2.5% ammonium molybdenate	200 µl
Aminonaphthol sulphonic acid solution	50 µl

The reaction was carried out at 37°C for 10 minutes, alongside phosphate standards. After cooling to room temperature, the absorbance at 600nm was read immediately against a blank. The amount of phosphate released in the sample was calculated from a standard curve (0.5 µmole of Pi gives an A_{600} of approximately 0.4).

ATPase activity was calculated as µmoles of inorganic phosphate released per minute per mg of mitochondrial protein in the diluted extract (determined by the Lowry method - Section 2.20).

2.20 PROTEIN DETERMINATION (LOWRY METHOD).

Protein concentration was determined using the method of Lowry (1951). The following solutions were used:

Solution A - 2% Na_2CO_3, 1.6% Na tartrate, 0.4% NaOH, 1% SDS.
Solution B - 1 ml 4% $CuSO_4$ and 100 ml Solution A.
Folin Reagent - diluted 1:1 with dH_2O

ASSAY

The sample to be assayed was diluted in dH_2O to a volume of 1 ml, alongside standards of BSA (0-100 μg/ml). To these, 3 ml Solution B was added. After 30 minutes, 1 ml diluted Folin reagent was added, and the absorbance at 660nm was read after a further 30 minutes. The concentration of protein in the sample was calculated from the standard curve.

2.21 DATABASE SEARCHES

DNA and protein databases were searched using the SEQNET facility provided by SERC Daresbury. Software used included the Univeristy of Wisconsin Genetics Computer Group Sequence Analysis Software Package Version 6.1. Searches were carried out using the FASTA program (Pearson and Lipman, 1988). Multiple sequence alignments were made using CLUSTAL, as described in the SEQNET User Notes. Databases searched were GenBank release 60.0, EMBL release 19.0 and SwissProt release 13.

CHAPTER THREE

OVEREXPRESSION OF F_1 β-SUBUNIT

3.1 INTRODUCTION

The targeting information which is sufficient to localize a mitochondrial precursor protein to the mitochondria is known to lie close to the amino terminus of the protein (Section 1.2.2). The nature of this information is at present unknown but it is not contained simply within the primary structure as presequences vary greatly between different mitochondrial precursors (Hurt and van Loon, 1986). There are several possible ways in which the presequence could exert its effect on the localization of the precursor: for example, the presequence could fold into a specific structure which can be recognized by either a receptor or a cytosolic factor; or the presequence could cause the precursor to adopt a translocation-competent structure. The purification of milligram amounts of a mitochondrial precursor protein would allow the interaction of the protein with other factors to be examined, and would also allow the structure of the presequence and precursor to be investigated.

A small quantity of the precursor of the β-subunit of the F_1-ATPase has been purified from yeast in a denatured form (Ohta and Schatz, 1984). A proportion of this purified precursor was able to renature and be imported into isolated mitochondria, thus indicating that it should be possible to purify import-competent precursor protein. Eilers and Schatz (1986) were able to purify sufficient quantities of a fusion protein consisting of the first 22 amino acids of cytochrome oxidase subunit IV (COXIV) fused to dihydrofolate reductase to perform binding experiments with isolated mitochondria. Pfaller and Neupert (1987) used the purified water soluble form of the mitochondrial outer membrane protein porin to investigate binding.

The purification of biochemically useful amounts of protein would be facilitated if it is present in the cell at a high concentration. Such an increase can be achieved by expressing the gene encoding the protein from a strong promoter while at the same time minimizing specific and non-specific proteolysis. This Chapter describes attempts to over-express the β-subunit

precursor protein in both *Escherichia coli* and *Saccharomyces cerevisiae* with a view to its purification. After purification, it should then be possible to identify other components which interact with the precursor during the targeting process and to investigate the structure of the precursor by a variety of biochemical and biophysical methods.

3.2 HIGH-LEVEL EXPRESSION OF THE *ATP2* GENE

Many expression systems have been developed for use in *Escherichia coli* and in the yeast *Saccharomyces cerevisiae* to express foreign and native proteins at high levels. Both types of system use the same principle: the gene encoding the protein to be expressed is sub-cloned behind a promoter which is highly efficient in the host organism; and the promoter fusion is introduced into the host by means of a self-replicating plasmid vector. The choice between using either bacterial or yeast systems depends on the particular protein, specifically in relation to its stability and function. The precursor form of the β-subunit of the F_1-ATPase is encoded by the *ATP2* gene in *S. cerevisiae*, and this gene has been cloned and sequenced (Saltzgaber-Muller *et al.,* 1983; Takeda *et al.,* 1985).

3.2.1. Expression of the β-subunit In *Escherichia coli*.

One advantage of using an *E. coli* system to express a mitochondrial precursor protein for subsequent purification is that *E. coli* is not thought to possess a protease which recognizes the cleavage site of mitochondrial precursors and therefore specific proteolytic degradation to the mature form can be eliminated. Also, *E. coli* does not possess a tough cell wall, and therefore extraction of protein from the cell is far more simple and efficient than with yeast.

The high-level expression of mitochondrial precursor proteins in *E. coli* has already been attempted by several groups. The precursor of rat ornithine transcarbamylase has been expressed in *E. coli* using the *tac* promoter to approximately 0.1% of total cell protein (Sheffield *et al.,* 1986). Similarly, the

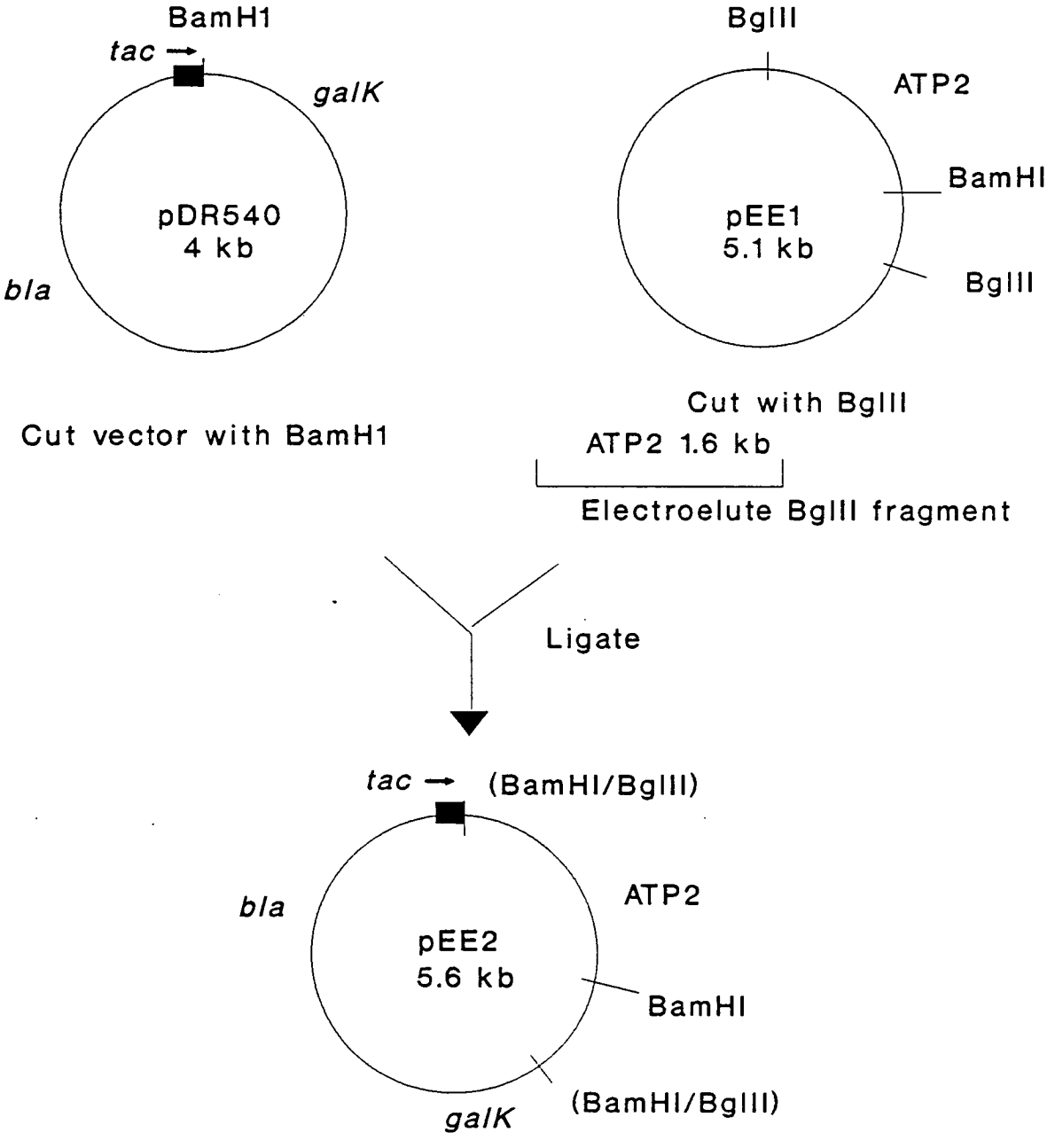
precursor of rat mitochondrial aspartate aminotransferase has been expressed using the *tac* promoter, to give 1% of total cell protein (Mattingley *et al.,* 1987). More recently, Taylor (1989) describes the high-level expression of cytochrome b_2 using the λp_L promoter (Crowl *et al.,* 1985).

3.2.1.1 Using the *tac* Promoter

The *tac* promoter is a hybrid promoter which contains DNA sequences corresponding to the -35 region of the *trp* promoter and the -10 region of the *lac* promoter. The presence of the latter region means that transcription can be derepressed by the addition of an analogue of lactose such as IPTG. The expression vector pDR540 (Russell and Bennett, 1982; Figure 2.1.) also contains a ribosome binding site (RBS) which should result in the efficient initiation of translation from the cloned gene's AUG translation start site. Also present are sequences for efficient termination of transcription downstream of the *galK* gene which is expressed with the cloned gene as a single operon.

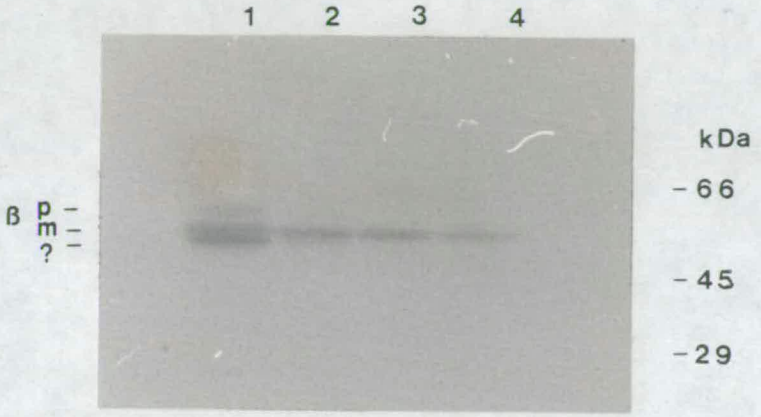
The coding region of the *ATP2* gene was sub-cloned into the vector pDR540 to give the recombinant expression vector pEE2 (Figure 3.1). Plasmids pEE2 and pDR540 were transformed into a *lon*$^-$ (protease deficient) strain of *E. coli*, KC1101. This strain also over-produces the LAC repressor protein, which allows expression from the *tac* promoter to take place only after the addition of excess IPTG. Cells were grown under repressing and derepressing conditions as described in the legend to Plate 3.1, and total cell proteins were analyzed by SDS-PAGE. Proteins were visualized by staining with Page Blue Electran but no difference in protein bands could be seen between the strain harbouring the parental plasmid (pDR540) or the recombinant plasmid (pEE2) under repressing or derepressing conditions. In order to detect any possible protein product of the *ATP2* gene, the proteins were transferred to a nitrocellulose membrane using the Western blotting procedure (Section 2.13). The blot was probed with antisera raised against the mature β-subunit and then with [^{125}I]-Protein A. The autoradiogram of the blot (Plate 3.1) reveals a band in all four lanes at 52,000 Daltons. This is

Figure 3.1 Construction of Plasmid pEE2 (*tac* Promoter)



The 1.6 kb BglII fragment containing the ATP2 gene from pEE1 (Figure 2.12) was ligated into the BamHI site of the plasmid pDR540 (Figure 2.1).

Plate 3.1 Expression of the *ATP2* Gene from the *tac* Promoter in *E. coli*



Strains of E. coli were grown to an OD_{600} of 0.3, and transcription from the tac promoter was induced by the addition of 70 µM IPTG, and growing the cells to stationary phase. Total cell protein was prepared and separated by SDS-PAGE. Proteins were transferred to nitrocellulose, probed with antisera raised against F_1 β-subunit, and bound antibody was detected using ^{125}I-protein A and autoradiography.

Lane	Strain
1	*KC1101 + pEE2 + IPTG*
2	*KC1101 + pEE2*
3	*KC1101 + pDR540*
4	*KC1101*

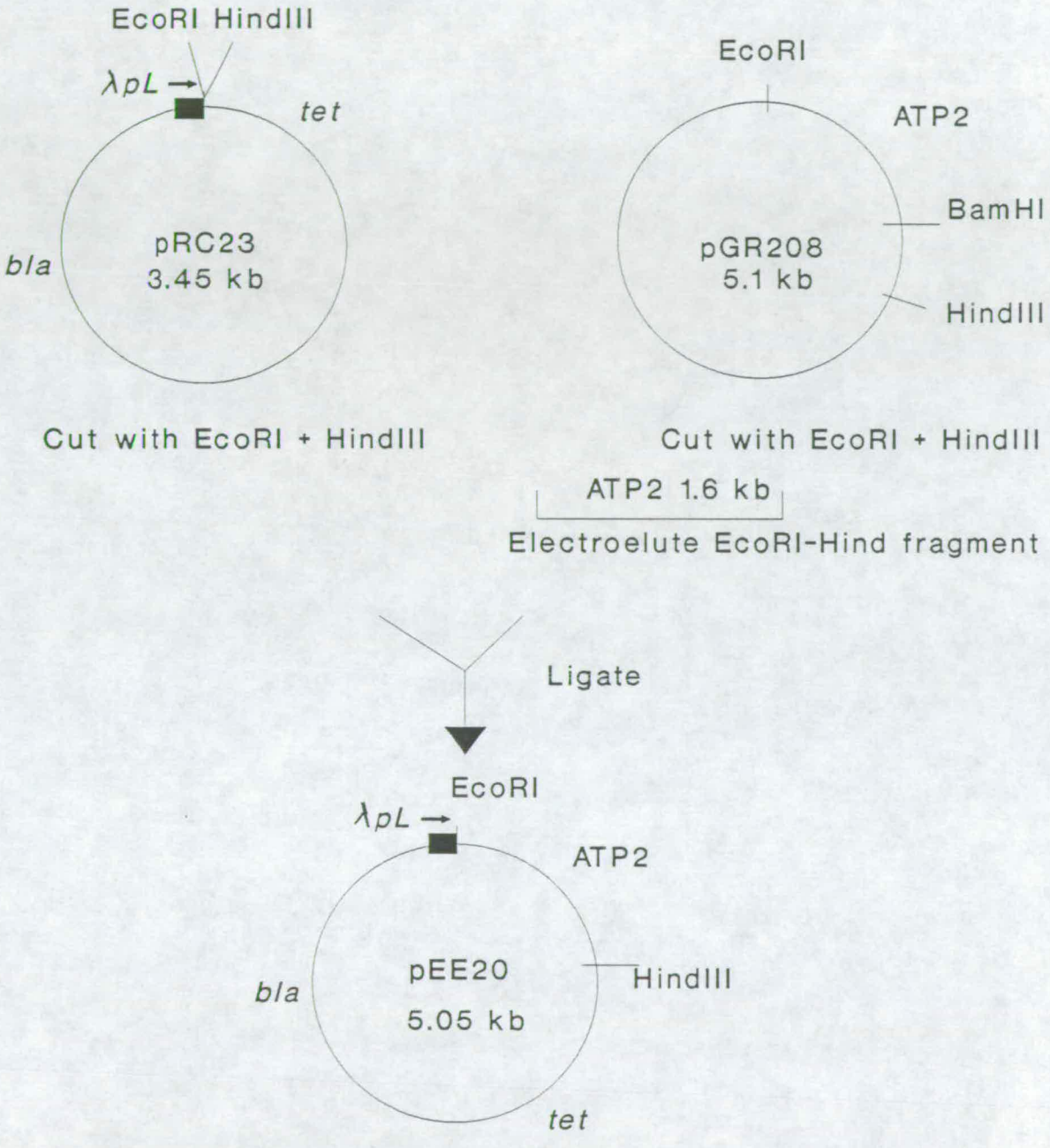
presumably the *E. coli* F_1 β-subunit which has strong homology with the yeast F_1 β-subunit at the protein level (Takeda *et al.,* 1985). A faint band is present at approximately 54,000 Daltons when expression of the *ATP2* gene is induced (Lane 1). This is presumably the *ATP2* gene product corresponding to the precursor form of the β-subunit. A lower band is also present in this lane and this could be the result of specific cleavage of the expressed gene product, to give a lower molecular weight protein; or it may be the product of an alternative translation start site. The concentration of the precursor form of yeast F_1 β-subunit is very low compared with the bacterial F_1 β-subunit which is present in the cell at about 0.2% of total cell protein (Futai and Kanazawa, 1983), and can therefore be estimated to constitute only 0.05% of total cell protein.

3.2.1.2 Using Bacteriophage λp_L Promoter

In an attempt to increase the level of protein expressed in *E. coli*, another expression system was used which utilizes the bacteriophage λp_L promoter in the expression vector pRC23 (Crowl *et al*, 1985; Figure 2.2). The λp_L promoter is one of the strongest bacterial or phage promoters known, and has the advantage of being tightly regulated so that transcription can be controlled using a temperature sensitive cI repressor (Rosenberg *et al*, 1983). This means that proteolysis may be minimized by expressing large amounts of protein over a short period of time, and also reduces any possible inhibitory effect that the expressed protein may have on cell growth. Plasmid pRC23 also contains a model ribosome binding site (RBS) which should enhance the efficiency of translation initiation.

The *ATP2* gene was subcloned behind the λp_L promoter and RBS in the vector pRC23 to give plasmid pEE20 as depicted in Figure 3.2. The recombinant plasmid was transformed into strain NF1 which contains the temperature sensitive cI repressor, and was therefore grown at 30°C. In order to express the *ATP2* gene from this promoter, the strains containing the plasmids pRC23 and pEE20 were grown in LB at 30°C to an OD_{600} of 0.3,

Figure 3.2 Construction of Plasmid pEE20 (λP_L Promoter)



The 1.6 kb EcoRI-HindIII fragment containing the ATP2 gene from pGR208 was ligated into the EcoRI-HindIII sites of the plasmid pRC23 (Figure 2.2).

and the incubation temperature was then shifted to 42°C. Samples were taken from the culture at time intervals, and proteins were extracted, separated by SDS-PAGE, transferred to a nylon membrane by Western blotting (Section 2.13) and the membrane was probed with antisera raised against β-subunit. The results (Plate 3.2) show that the expression from the plasmid pEE20 of several proteins with antigenic cross-reactivity to β-subunit is induced within 30 minutes of the cells being shifted to 42°C. A faint band can be seen in all the lanes which corresponds in size to the β-subunit precursor. One of the strongest bands is approximately the same size as the mature form of the β-subunit from yeast (Lane 10). Also another lower molecular weight protein band is present which appears to be the same size as that seen with the *tac* promoter after only 1 hour of derepression (Plate 3.1). These bands may be a result of proteolysis of the protein, but may also be due to initiation of translation at an alternative AUG. The latter is a more likely explanation given the almost constant ratio of the two bands over induction time - one might expect a specific proteolysis event to give rise to a greater amount of the processed form as time progressed. A similar result was obtained by Sheffield *et al.* (1986) who observed a lower molecular weight band when attempting to express rat pre-ornithine transcarbamylase in *E. coli* from the *tac* promoter.

The amount of protein expressed from the λp_L promoter accumulates to some extent after 16 hours of growth, but is not very high compared to the levels obtained with another yeast mitochondrial protein cytochrome b_2 expressed using the same system (Taylor, 1989). The fact that both the *tac* promoter and the λp_L promoters give a lower level of expression than expected suggests that it is the same problem in both cases which is affecting expression of the *ATP2* gene in *E. coli*.

3.2.1.3 Possible Causes of Low Level Expression in *E.coli*.

Reasons for the low level expression achieved fall into three categories: transcriptional, translational, and post-translational. As the transcription of

Plate 3.2 Expression of the *ATP2* Gene from λp_L Promoter in *E.coli*.

Strains of E. coli were grown at 30°C to an OD_{600} of 0.5, and transcription from the λp_L promoter was induced by shifting the cultures to 42°C. Samples were taken at time intervals, proteins prepared and a Western blot of electrophoresed proteins was probed with antisera against β-subunit. Bound antibody was detected with [^{125}I]-protein A and autoradiography.

Lane	Strain	Time after Induction
1.	*NF1 + pRC23*	*0 min*
2.	*NF1 + pRC23*	*1 hour*
3.	*NF1 + pRC23*	*14 hours*
4.	*NF1 + pEE20 (ATP2)*	*0 min*
5.	*NF1 + pEE20 (ATP2)*	*30 mins*
6.	*NF1 + pEE20 (ATP2)*	*1 hour*
7.	*NF1 + pEE20 (ATP2)*	*3 hours*
8.	*NF1 + pEE20 (ATP2)*	*6 hours*
9.	*NF1 + pEE20 (ATP2)*	*14 hours*
10.	*SF747-19D Total Yeast Protein*	

other genes using these same promoters is known to be efficient (Taylor, 1989), transcriptional effects were not pursued. It is possible that termination of transcription of the *ATP2* gene is premature due to the presence of termination sequences which the bacterial cell might recognize. However no consensus rho-dependent or rho-independent terminators were apparent in the DNA sequence 3' to the transcription start site. Although transcription and translation are linked both temporally and spatially in bacterial systems, some degradation of mRNA may occur due to inherent instability of the message, and this would also lead to a reduction in the amount of protein produced.

At the level of translation there are many possible problems which may arise. Firstly, the initiation of translation depends on an efficient RBS, which both the expression vectors supply. However, as mentioned in Section 3.2.1.2, there may well be initiation at a second AUG downstream due to a fortuitous RBS, which may be interfering with the ribosome binding to the RBS upstream. This may result in an overall reduction in the amount of protein expressed. Secondly, the spacing between the RBS and the initiating AUG of the *ATP2* gene is far from optimal at 20 nucleotides, as it is usually around 7 to 9 nucleotides for most *E. coli* genes (Gold and Stormo, 1987). Thirdly, the codon preference of *E. coli* may not allow efficient translation of the *ATP2* gene, due to a low abundance of the tRNA species which may be required for the translation of some codons in the *ATP2* gene. The codon bias of the yeast *ATP2* gene was calculated for expression in *E. coli* (Table 3.1) and compared to that for highly expressed bacterial genes (Table 3.2.) (Bennetzen amd Hall, 1982). The codon bias index of the *ATP2* gene is of a value comparable to that of the *lacI* gene which codes for the LAC repressor protein and which is only expressed at very low levels in the cell. Therefore the codon bias may be an important influence on the over-expression of the *ATP2* gene.

Post-translational problems include the possibility that there may be extensive proteolysis of the synthesized protein which may not be overcome by its overexpression, even though it is expressed from a regulated promoter

Table 3.1 Calculation of the Codon Bias Index for the *S. cerevisiae ATP2* gene when expressed in *E. coli*.

Amino Acid	Preferred Codon in E. coli[a]	Usage of Codon in ATP2	Total No in ATP2	Random Expectation of Usage[b]
ALA	No preference	.	.	.
SER	UCU, UCC	22	33	16.5
THR	ACU, ACC	25	32	16
VAL	GUU, GUA	21	45	22.5
ILE	AUC	12	34	11.33
ASP	GAC	9	26	13
PHE	UUC	7	17	8.5
TYR	UAC	4	12	6
CYS	No preference	.	.	.
ASN	AAC	7	11	5.5
HIS	CAC	1	8	4
GLU	GAA	35	37	18.5
GLY	GGU, GGC	42	46	23
GLN	CAG	1	21	10.5
LYS	AAA	14	28	14
PRO	CCG	3	27	6.75
LEU	CUG	0	48	8
ARG	AGA	18	28	4.67
MET	AUG[c]	.	.	.
TRP	AGG[c]	.	.	.
	TOTAL	X = 221	Y = 453	Z = 188.76

CODON BIAS INDEX

$$= \frac{\text{(usage of preferred codons) - (random expectation)}}{\text{(total no. of residues) - (random expectation)}}$$

$$= \frac{X - Z}{Y - Z} = \frac{221 - 188.76}{453 - 188.76}$$

$$= \mathbf{0.12}$$

[a] Calculated for the highly expressed *E. coli* genes *ompA, lpp, tufA and tufB.* (Bennetzen and Hall, 1982).

[b] The random expectation of usage is the number of residues in the protein of the particular amino acid multiplied by the fraction of all codons for that amino acid which are 'preferred' (eg for LEU - 48 x 1/6 = 8).

[c] Not degenerate therefore not used in calculation.

Table 3.2 Comparison of the Codon Bias Index of the *ATP2* Gene with those of Highly Expressed *E. coli* Genes

Gene	Codon Bias Index[a]	Protein Molecules Per Cell[a]
E. coli lpp	0.84	750,000
E. coli tufA	0.84	200,000 - 1,000,000
E. coli tufB	0.81	200,000 - 1,000,000
E. coli ompA	0.78	200,000
E. coli lacI	0.18	10
S. cerevisiae ATP2	0.12	Not determined

[a]Data obtained from Bennetzen and Hall 1982, except for *ATP2* gene where Codon Bias Index is calculated in Table 3.1

(Cheng *et al.*, 1981) as well as the possible specific cleavage (Plates 3.1, 3.2). Also, high levels of the yeast F_1 β-subunit in the bacterial cell may have a detrimental effect on cell growth - it may for example interact with the F_1 portion of the bacterial ATPase and render it non-functional; or the targeting signal of the precursor may have some adverse effect in *E. coli*. The high level expression of other foreign proteins in *E. coli* has been shown to be toxic to the host cell and therefore there may be a strong selection against high plasmid copy number in the cell giving rise to instability in the plasmid, and such a phenomenon has been observed with other genes (Nichols and Yanofsky, 1983).

The low level of expression obtained, and the uncertainty of the site of translational initiation meant that the use of *E. coli* as a host in which to express the *ATP2* gene seemed unlikely to yield sufficient precursor protein to allow purification of useful amounts of the protein for further studies. For this reason, yeast expression systems were examined.

3.2.2 Expression of the β-subunit in *S. cerevisiae*

Some of the problems encountered with the *E. coli* expression sytems may be overcome by expressing the protein in yeast; for example, the codon bias in yeast might be more in favour of high level expression; or the yeast translation machinery might recognize only the correct initiating AUG codon; or the protein may be more stable in the cytoplasm of yeast than *E. coli*. On the other hand, yeast possesses the matrix protease which cleave mitochondrial precursor proteins to the mature form.

Several yeast expression vectors are used, all of which can be propagated in *E. coli* and maintained in yeast. These vectors each contain different strong yeast promoters, two of which are constitutive (*ADH* and *PGK*) and one of which is inducible (*PHO5*). The advantages of an inducible promoter are that degradation of the expressed protein can be minimized if it is expressed over a short period of time. However, higher overall yields can often be obtained with some constitutive promoters.

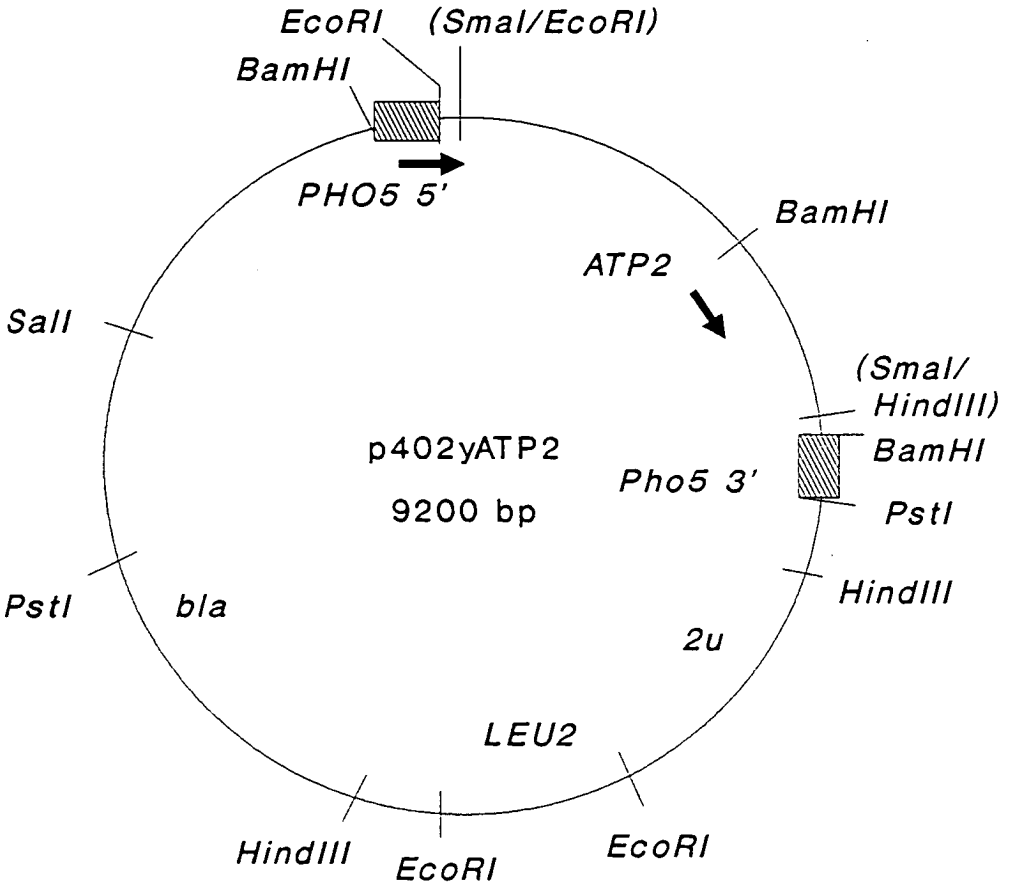
3.2.2.1 Expression from the *PHO5* Promoter

The *PHO5* promoter is a strong inducible promoter from the gene encoding acid phosphatase (Meyhack *et al* 1982). This promoter is induced at low concentrations of inorganic phosphate in the growth medium. The promoter and transcription termination sequences are contained on plasmid p31y (Figure 2.3). The recombinant expression vector p402yATP2 is shown in Figure 3.3. The yeast strains SF747-19D and GR1 were transformed with p402yATP2, and single isolates (EMY1 and EMY4 respectively) were grown up under repressing (0.1% KH_2PO_4) and non-repressing conditions (no KH_2PO_4) as described in the legend to Plate 3.3. The proteins were analyzed by SDS-PAGE as described previously, and the amount of F_1 β-subunit present was compared to wild-type levels by Western blotting (Section 2.13), (Plate 3.3). It is apparent that expression of the β-subunit protein has been increased using the *PHO5* promoter to approximately 5-fold higher than wild-type levels. However, over-expression is only achieved in late log phase cultures. Unfortunately, the precursor protein seems to be rapidly processed under these growth conditions, and only a small percentage of protein remains as the precursor form. As the immediate aim is to purify this precursor form, it would be advantageous to be able to express the protein at an earlier growth phase in the hope that the amount of precursor processed could be minimized. This could be achieved either by using a constitutive promoter such as that found in front of the *PGK* gene or *ADH1* gene; or by using an inducible promoter which can be induced during exponential growth such as that found in front of the *GAL1* gene.

3.2.2.2 Expression from the *PGK* Promoter

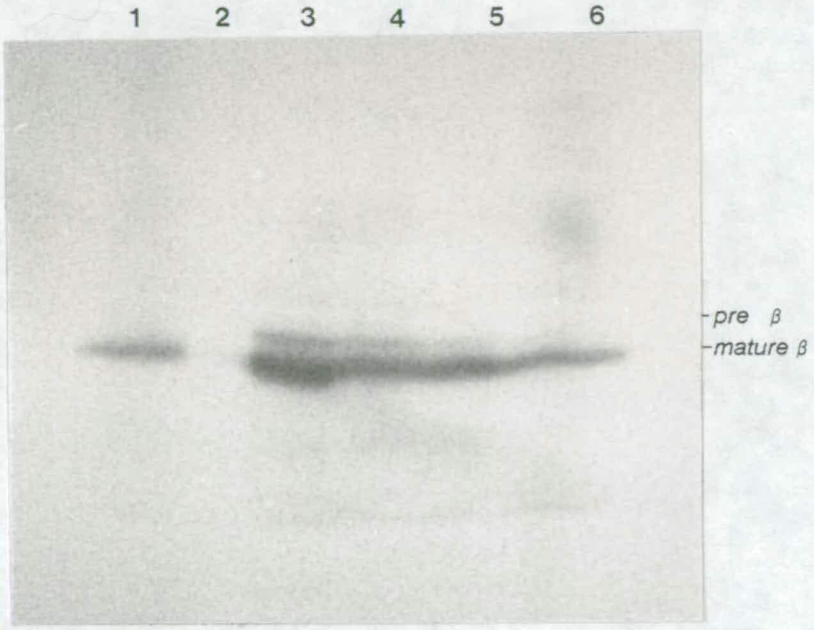
The *PGK* gene codes for phosphoglycerate kinase, a key enzyme in the glycolytic pathway which is expressed at very high levels during exponential growth on fermentable carbon sources. The *PGK* promoter has been described and used for the overexpression of mammalian proteins in yeast (Dobson *et al*, 1983). The *ATP2* gene was subcloned into the expression

Figure 3.3 Plasmid p402yATP2 (*PHO5* Promoter)



Plasmid p402yATP2 was constructed by B. Stevenson and M. Walker by inserting the end-filled 1.6 kb EcoRI-HindIII fragment containing the ATP2 gene from pGR208 (Figure 2.12) into the filled-in SmaI site of p31y (Figure 2.3). The 3.3 kb HindIII fragment containing the LEU2 gene and 2μ origin of replication from plasmid pMA91 was inserted into the HindIII site of p31y.

Plate 3.3 Expression of the *ATP2* Gene from the *PHO5* Promoter in *S. cerevisiae*



Total yeast protein (20 µg) was separated by SDS-PAGE, and was transferred to nitrocellulose membrane. The membrane was probed with antisera against F_1 β-subunit, and detected with ^{125}I protein A and autoradiographed.

Lane Strain

1. *SF747-19D*
2. *GR1*
3. *GR1 + p402yATP2 grown in Low Pi medium, OD_{600} = 1.4*
4. *GR1 + p402yATP2 grown in High Pi medium, OD_{600} = 1.4*
5. *GR1 + p402yATP2 grown in Low Pi medium, OD_{600} = 0.5*
6. *GR1 + p402yATP2 grown in High Pi medium, OD_{600} = 0.5*

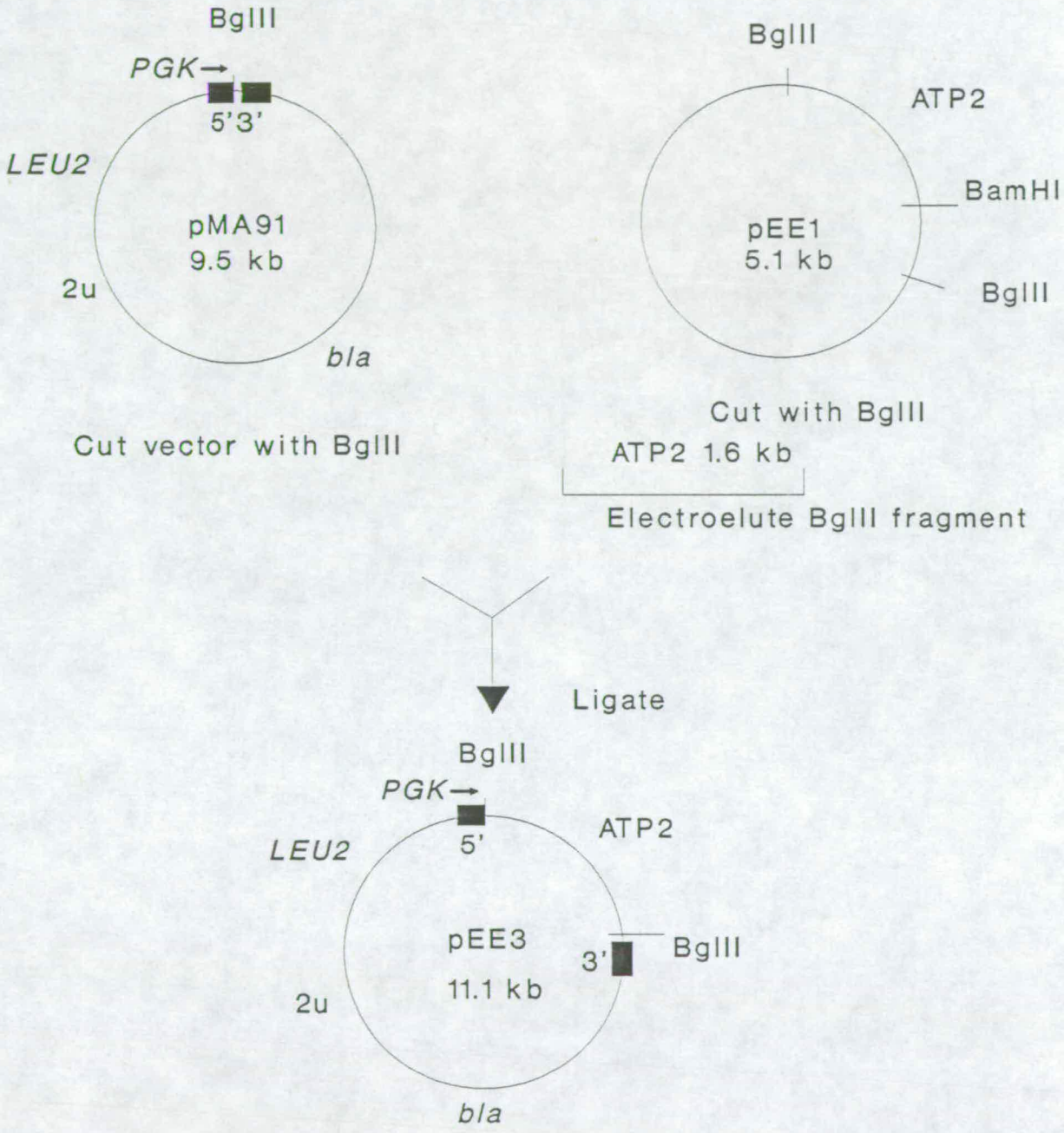
vector pMA91 (Mellor *et al.,* 1983; Figure 2.4) to give the recombinant plasmid pEE3 (Figure 3.4.). The plasmid was transformed into SF747-19D to give strain EMY5 and into GR1 to give EMY6. When strains EMY5 and EMY6 were streaked on YPDG (limiting glucose) plates, they appear to have identical growth characteristics to GR1 which is disrupted for the *ATP2* gene and is therefore phenotypically petite (Plate 3.4). This result was observed with several independent transformants. It is possible that the plasmid in EMY6 was incapable of complementing the *ATP2* gene disruption in strain GR1. However, this could not explain the petite phenotype of EMY5.

A Western Blot of protein from EMY6 and SF747-19D shows that there is a considerable increase in the amount of F_1 β-subunit expressed in EMY6 compared to wild-type levels, approximately 8-10 fold (Plate 3.5.). This means that the petite phenotype cannot be due to an insufficiency of the F_1 β-subunit protein. Also, the presence of a higher molecular weight band suggests that the precursor form is accumulating to some extent. This could be caused by saturation of a step in the targeting pathway, for example translocation or processing and might explain why both EMY5 and EMY6 have a petite phenotype: perhaps other precursor proteins which are required for efficient respiration or growth are unable to be translocated or processed at a rate commensurate with their use.

3.2.2.3 Expression from the *ADH1* Promoter

The *ADH1* gene codes for alcohol dehydrogenase 1, an enzyme which is expressed at constitutively high levels during exponential growth. The *ATP2* gene was subcloned between the *ADH1* promoter and terminator in the vector pVT102A (Vernet *et al.,*1987; Figure 2.5) to give the recombinant plasmid pEE4 (Figure 3.5). The plasmid pEE4 was transformed into the yeast strain SF747-19D to give strain EMY11. Expression of the F_1 β-subunit was measured as previously described by comparison to wild type levels using antibody detection methods. Results indicate (Plate 3.6.) that over-expression of the *ATP2* gene is achieved from the *ADH* promoter, but without the

Figure 3.4 Construction of Plasmid pEE3 (*PGK* Promoter)



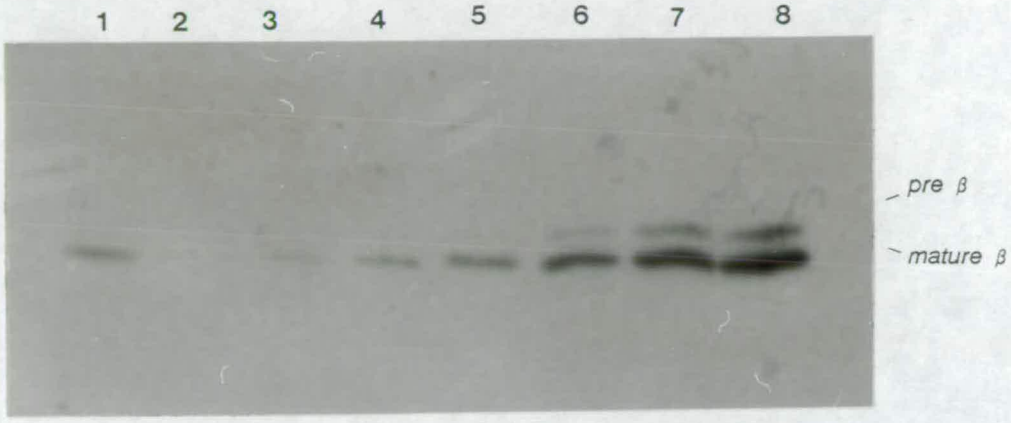
The 1.6 kb BglII fragment containing the ATP2 gene from pEE1 (Figure 2.12) was ligated into the BglII site of the plasmid pMA91 (Figure 2.4).

Plate 3.4 Growth of Strains on Limiting Glucose Plates

Strains were streaked out onto a YPDG plate, and grown at 30°C for 3 days.

Strain	Promoter Fusion
1. SF747-19D	*Wildtype ATP2*
2. GR1	*No ATP2*
3. EMY11 (SF747-19D + pEE4)	*ADH::ATP2*
4. EMY4 (GR1 + p402yATP2)	*PHO5::ATP2*
5. D273-10B-ρ^-	*WIld type ATP2, mitochondrial petite*
6. EMY5 (SF747-19D+pEE3)	*PGK::ATP2*
7. EMY6 (GR1+pEE3)	*PGK::ATP2*

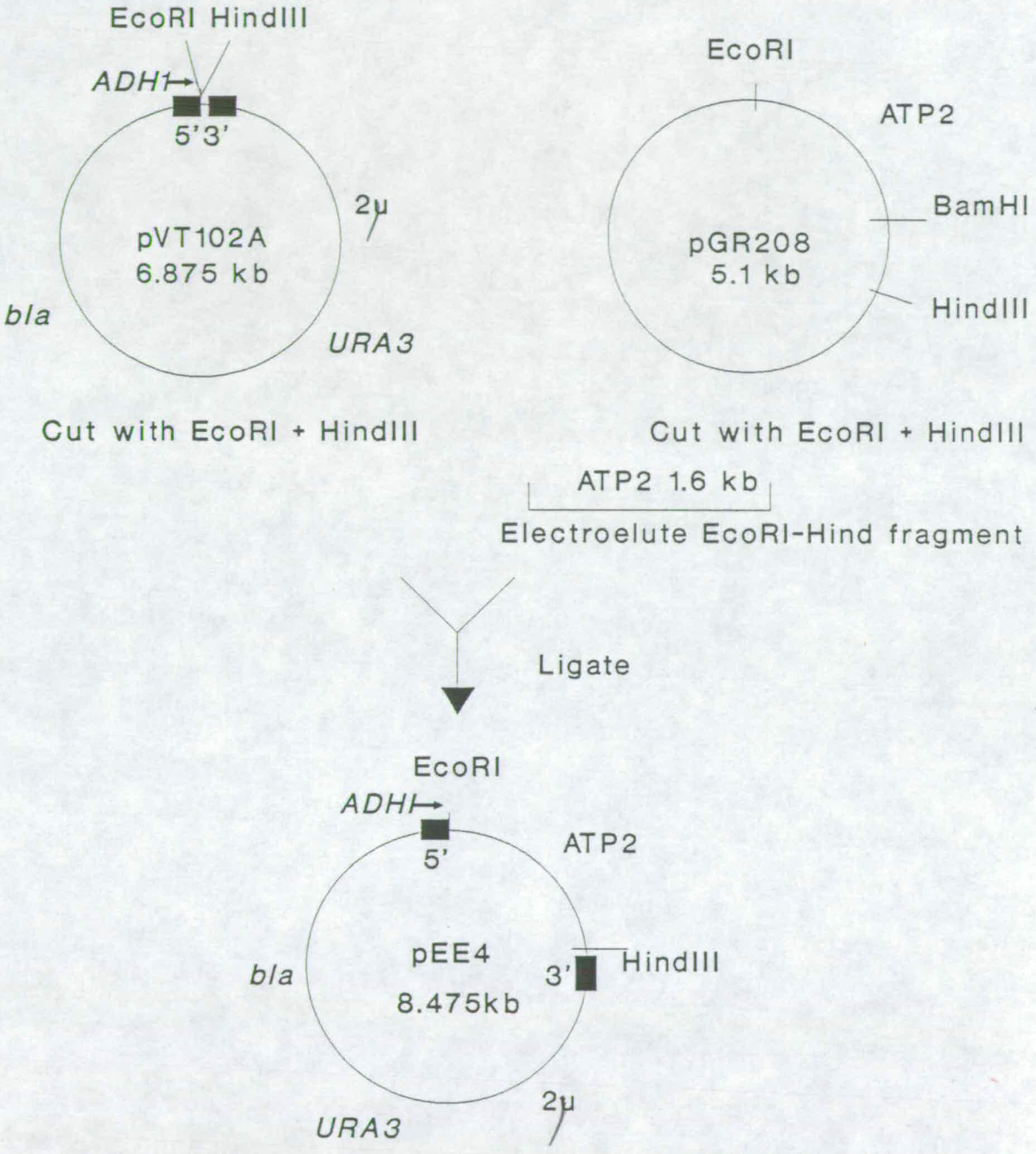
Plate 3.5 Comparative Quantitation of Overexpression of β-subunit from the *PGK* Promoter in *S. cerevisiae*.



Protein was prepared from SF747-19D (wild-type) grown in 2% lactate, and EMY6 (GR1+pEE3, PGK::ATP2) grown in SD media to an OD_{600} of 0.7. The amount of protein in the extracts was measured. Samples containing equal amounts of protein and dilutions were analyzed by Western blot, probed with antisera against β-subunit and detected with [^{125}I]-protein A and autoradiography.

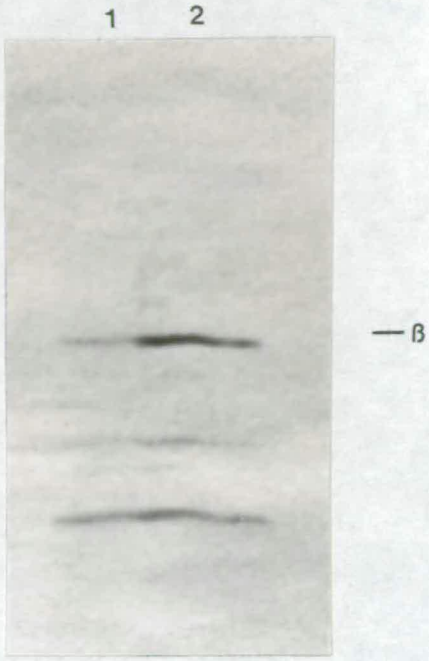
Lane	Sample	Amount of Protein (μg)	Dilution
1	SF747-19D	20	1 x
2	EMY6	0.312	64 x
3	EMY6	0.625	32 x
4	EMY6	1.25	16 x
5	EMY6	2.5	8 x
6	EMY6	5	4 x
7	EMY6	10	2 x
8	EMY6	20	1 x

Figure 3.5 Construction of Plasmid pEE4 (*ADH* Promoter).



The 1.6 kb EcoRI-HindIII fragment containing the ATP2 gene from pGR208 was ligated into the EcoRI-HindIII sites in the polylinker of the plasmid pVT102A (Figure 2.5).

Plate 3.6 Expression of the *ATP2* Gene from *ADH1* promoter



Yeast strains were grown to an OD_{600} of 0.7 in SD medium. Proteins were extracted and ß-subunit was detected by Western blotting.

Lane	Strain	Promoter Fusion
1	*SF747-19D*	*ATP2 Wild type*
2	*EMY11*	*ADH1::ATP2*

accumulation of precursor protein under normal growth conditions. When compared to the amount of protein expressed from the *PGK* promoter, it is apparent that the *ADH* promoter does not yield quite as high levels of protein (data not shown). However, strain EMY11 does not have a petite phenotype, and therefore the *ADH* expression vector may be useful if the precursor form could be accumulated in this strain.

3.3 LOCALIZATION OF PRECURSOR

It was noticed that when the β-subunit was expressed at high levels from the *PHO5* or *PGK* promoter, a band of higher molecular weight than the mature protein could also be detected on Western Blots probed with antisera against the β-subunit (Plate 3.5). This band runs in a position similar to where the precursor form of the protein would be expected to run.

There are two possible reasons to account for the fact that this precursor protein is not processed to the mature form and could therefore be detected. Firstly, the precursor protein could be present in large enough amounts to saturate the binding or translocation step, resulting in some of the precursor protein remaining on the cytoplasmic side of the mitochondrial membrane. Secondly, the precursor protein could be transported efficiently, but the processing step within the matrix might be limiting. In this case, there would be an accumulation of precursor within the mitochondrial matrix. It is possible to differentiate between these two alternatives by isolating mitochondria from the strain which overexpresses the precursor protein and determining whether the precursor is associated with the mitochondria or with the cytoplasmic fraction.

Cells of strains EMY6 and SF747 were broken by lysis of sphaeroplasts, and the mitochondria were separated by differential centrifugation from the lysate (Section 2.16). Cell equivalent amounts of the mitochondrial protein, post-mitochondrial supernatant protein and total sphaeroplast protein from EMY6 and SF747 were separated by SDS-PAGE, transferred to a membrane by Western Blotting, and the membrane was probed with antisera raised

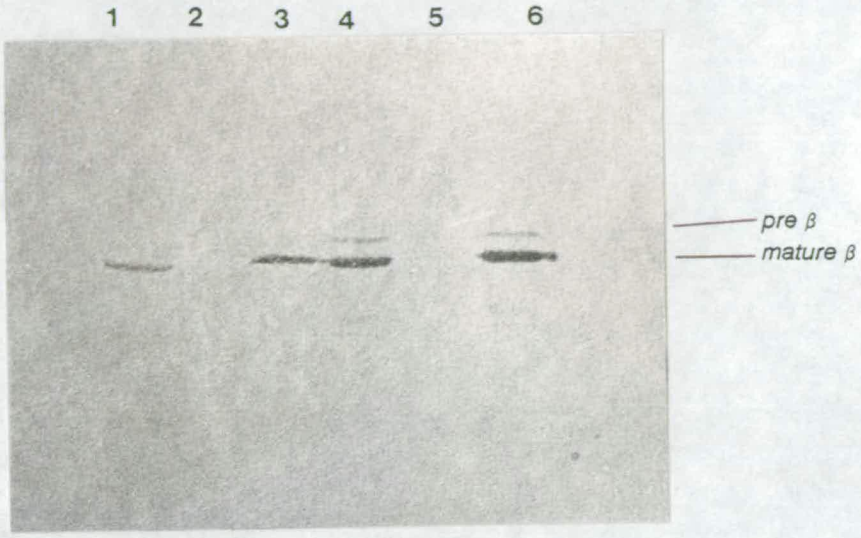
against the β-subunit (Plate 3.7). This reveals the presence of two bands in the mitochondrial and sphaeroplast fractions of EMY6. It is clear that there is no precusor band in the post-mitochondrial supernatant fraction and that all the detectable precursor is associated with the mitochondrial fraction. This suggests that there is no accumulation of precursor in the cytoplasm and that the step which is saturated is mitochondrially-located. It is possible that components of the protein translocating machinery are not expressed at very high levels during exponential growth on glucose, and that these steps are saturable under these conditions.

3.4 ACCUMULATION OF PRECURSOR

To be able to purify the precursor form of the β-subunit, it would be advantageous to prevent the majority of the overexpressed protein from being processed to the mature form. The optimal results with strain EMY6 show that only approximately 20% of the total antigen is present as the precursor protein (Figure 3.5) and it was hoped that this could be increased to about 50% precursor by the prevention of import. Reid and Schatz (1982a) have shown that the addition of carbanyl cyanide *m*-chlorophenylhydrazone (CCCP) to actively growing yeast cells causes the accumulation of some mitochondrial precursors in the cytoplasm. The abolition of the electrochemical potential by this chemical is thought to cause the block in import (Section 1.4.4). This effect has also been shown to be more pronounce in ρ- cells which are defective in oxidative phosphorylation.

The concentration of CCCP causing a slight inhibition in growth was determined as 20 μM (data not shown). The accumulation of β-subunit precursor in strain EMY4 after the addition of 20 μM CCCP was determined by taking 50 ml samples at various time points from a 1 litre culture to which CCCP had been added, extracting total cell protein from the cells, separating by SDS-PAGE, Western Blotting and probing with antisera against β-subunit. (Plate 3.8). It is apparent that the amount of precursor does not seem to increase after the addition of CCCP.

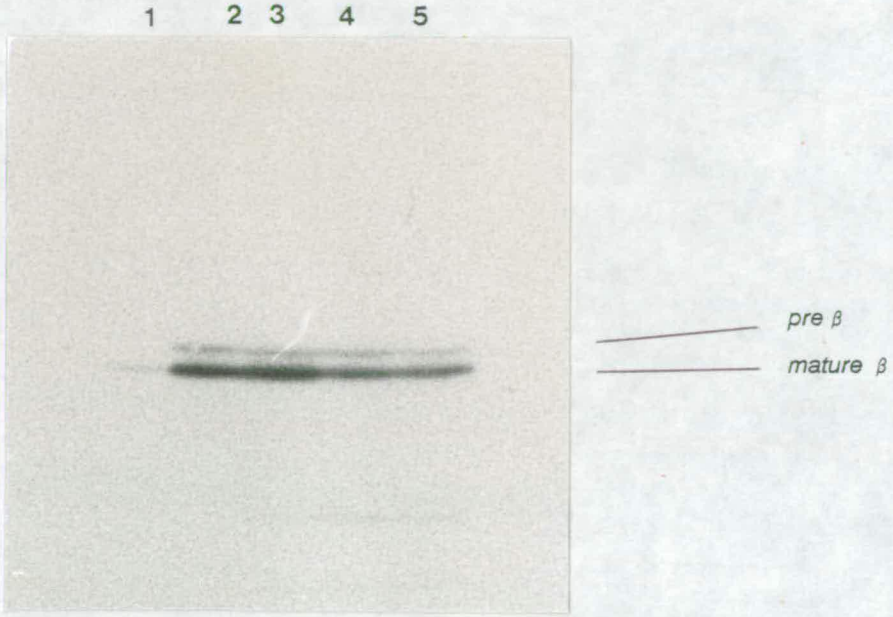
Plate 3.7 Subcellular Localization of Precursor F_1 β-subunit in Wild type and Overexpressing Strains



Mitochondria were isolated by differential centrifugation of lysed sphaeroplasts, and the post-mitochondrial supernatant was kept. Cell equivalents of proteins (20 µg Total and Supernatant, 2 µg Mitochondrial) were analyzed by SDS-PAGE and Western blot using antisera against β-subunit.

Lane	Strain	Fraction
1.	SF747-19D	Total lysate
2.	SF747-19D	Postmitochondrial supernatant
3.	SF747-19D	Mitochondrial
4.	EMY6 (PGK::ATP2)	Total lysate
5.	EMY6 (PGK::ATP2)	Postmitochondrial supernatant
6.	EMY6 (PGK::ATP2)	Mitochondrial

Plate 3.8 Effect of CCCP on Accumulation of Precursor.



Yeast were grown in SD to an OD_{600} of 0.3. CCCP was added to a concentration of 20 μM, and cells grown for up to 8 hours. Samples were taken at various time points, and protein extracts were made. Approximately 20 μg protein was loaded onto an SDS gel prior to western blotting.

Lane	Strain	Time
1	SF747-19D	0
2	EMY6 (PGK::ATP2)	0
3	EMY6 (PGK::ATP2)	2 hours
4	EMY6 (PGK::ATP2)	4 hours
5	EMY6 (PGK::ATP2)	8 hours

3.5 DISCUSSION

In this Chapter the possibility of overexpressing the β-subunit of the F_1 ATPase in both yeast and bacterial systems with a view to the purification of the precursor form of the protein has been examined. Expression from the bacterial *tac* and λp_L promoters was investigated, and both gave rise to the production of proteins other than the full-length precursor protein. These were presumably due to translation initiation at other AUG codons.

Expression in yeast was examined using three different promoters, the most efficient of which was *PGK* promoter. Although a higher level of expression than with the wild-type promoter was observed, most of the precursor is processed to the mature form, and high levels appear to have an effect on the physiology of the cells. Localization studies showed that the precursor form is however associated with the mitochondria and this could enhance purification to some extent. An increase in precursor form was not observed when yeast cells were grown in the presence of CCCP.

Using the expression from the PGK promoter, it should be possible to purify the precursor form of the β-subunit from the mitochondria where it is present as about 5% of total mitochondrial protein. However, there seems to be some proteolytic degradation of the protein even when expressed at this high level and this could be overcome by using a regulated high level expression vector such as the galactose-inducible *PGK* promoter. In order to purify the precursor form however, much work needs to be done in optimizing growth conditions. Various conditions were tested and best yields were obtained by growing the cells in 2% glucose media to the start of exponential phase growth. This is presumably because the mitochondria are neither as abundant nor as well developed under these conditions, and therefore the rate at which the precursor form is synthesized can exceed the rate at which it is processed.

CHAPTER FOUR

IN VIVO AND *IN VITRO* IMPORT OF MUTANT PRECURSORS OF THE *β*-SUBUNIT PROTEIN.

4.1. INTRODUCTION.

The importance of a targeting sequence for the correct cellular localization of a protein has been demonstrated in a wide variety of biological systems, both eukaryotic and prokaryotic (Silhavy *et al.,* 1983). In the case of mitochondrial protein targeting, the amino terminus of a precursor has been shown to be sufficient to direct a fusion protein to mitochondria *in vivo* (Douglas *et al.,* 1984) and *in vitro* (Hurt *et al.,* 1984) (see section 1.4.2). In conjunction with this approach for defining presequences, the removal of regions within the presequence has given information on the requirement of a particular stretch of amino acids for correct and efficient localization. The effect of deletions in the presequence of a fusion protein of subunit IV of yeast cytochrome oxidase and dihydrofolate reductase has been studied *in vitro* and *in vivo* (Hurt *et al.,* 1985). The targeting capability of deletion mutants within the 32 residue leader peptide of the full length precursor of human ornithine transcarbamylase has been studied in an *in vitro* system (Horwich *et al.*, 1986).

One model of the targeting process proposes that the presequence is able to form a particular structure, and that this structure is then able to interact with components of the targeting machinery at various steps. Therefore when examining the nature of targeting information it is important not only to define which features of the precursor are required for targeting but also to discover at which stage these features are involved. In this respect, factors which affect the relative efficiency of targeting may yield information on the interaction between precursors and import components.

At the onset of this study, very little work had been carried out on the relative efficiency of targeting of various regions of the presequence of the F_1 ATPase β-subunit precursor, either *in vitro* or *in vivo*. The region of the β-subunit which is sufficient for targeting mouse DHFR to mitochondria has been defined as the first 15 amino acids using an *in vitro* system (Walker *et al.,* 1990). As the use of some fusion proteins has been shown to affect the

targeting ability of a presequence (Emr *et al.,* 1986), studies which focus on the efficiency of the targeting process would more accurately represent the biological situation if full length precursors were used. More recent work on the targeting of some deletion mutations of the β-subunit presequence using the wild-type protein has been described by Vassarotti *et al.* (1987b) and Bedwell *et al.* (1987). However these studies were carried out *in vivo* systems only, and the sensitivity of the assay relied solely on the ability of a yeast strain harbouring the β-subunit deletion protein to grow on an non-fermentable carbon source.

This Chapter describes the construction of deletions in the presequence of the β-subunit, and the examination of the relative efficiency of targeting of these mutant proteins both *in vivo* and *in vitro*, in an attempt to define regions which may be required for particular steps in the targeting process.

4.2 Construction of Deletion Mutations in the Presequence of β-subunit

To define features of the β-subunit precursor which are involved in its interaction with other proteins, stretches of amino acids were removed from the amino terminus of the protein by deleting the corresponding DNA from the *ATP2* gene. Three mutations were made resulting in the deletion of amino acids Δ2-15, Δ16-35 and Δ2-35 from the β-subunit presequence (Figure 4.1). All deletions were made by restriction endonuclease digestion, and religation as described in Section 2.7. The deletions were confirmed by DNA sequence analysis.

To examine the effect of amino acid deletions on the efficiency of import, two assay systems were used. An *in vitro* system allows the import process to be dissected into separate steps (e.g. binding, translocation) and may therefore give more information on how a particular deletion affects the efficiency of import. However, an *in vivo* system was also used as this will give a more accurate biological picture of mitochondrial targeting, as it allows the fate of a mutant protein within the cell to be examined.

Figure 4.1 Nucleotide Sequences of Wild Type and Deletion Precursors of the F_1-ATPase β-subunit, and the Deduced Amino Acid Sequence.

```
                                                +           +                   +           +       +
      EcoRI                                     Met Val Leu Pro Arg Leu Tyr Thr Ala Thr Ser Arg Ala Ala Phe Lys Ala Ala Lys Gln Ser Ala Pro Leu
WT    GAATTCAGATCTGTAGAGAAAACAAAAATAAAAAAAAGA ATG GTT TTG CCA AGA CTA TAT ACT GCT ACA TCC CGT GCT GCT TTT AAA GCA GCC AAA CAA TCC GCT CCG CTT
Δ16-35 GAATTCAGATCTGTAGAGAAAACAAAAATAAAAAAAAGA ATG GTT TTG CCA AGA CTA TAT ACT GCT ACA TCC CGT GCT GCT TTT ... ... ... ... ... ... ... ... ...
Δ2-15 GAATTCATCG..............................  ATG ... ... ... ... ... ... ... ... ... ... ... ... ... ... AAA GCA GCC AAA CAA TCC GCT CCG CTT
Δ2-35 GAATTCATCG..............................  ATG ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
No.                                             1   2   3   4   5   6   7   8   9   10  11  12  13  14  15  16  17  18  19  20  21  22  23  24

                                  +   +       +
      Leu Ser Thr Ser Trp Lys Arg Cys Met Ala Ser Ala Ala Gln Ser Thr Pro Ile Thr
WT    CTA TCC ACT TCG TGG AAA AGA TGT ATG GCC TCA GCT GCT CAA TCT ACT CCA ATC ACC
Δ16-35 ... ... ... ... ... ... ... ... CCA TCG ATG GCT GCT CAA TCT ACT CCA ATC ACC
Δ2-15 CTA TCC ACT TCG TGG AAA AGA TGT ATG GCC TCA GCT GCT CAA TCT ACT CCA ATC ACC
Δ2-35 ... ... ... ... ... ... ... ... ... ... ... GCT GCT CAA TCT ACT CCA ATC ACC
No.   25  26  27  28  29  30  31  32  33  34  35  36  37  38  39  40  41  42  43
```

KEY

\+ - positive amino acids

Underlined nucleotides indicate a deviation from the wild type sequence due to the introduction of an oligonucleotide linker in order to create restriction sites. The corresponding amino acid sequence in Δ16-35 would be **Pro Ser Met**.

4.3 *In vitro* Import of Mutant Precursors

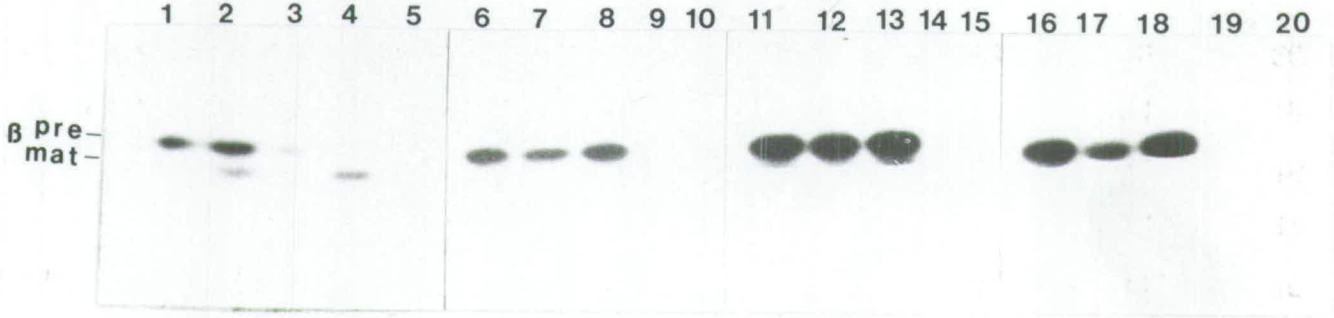
To test the efficiency of targeting to the mitochondria of the three mutant proteins (Δ2-15, Δ16-35, Δ2-35) compared with the wild-type protein, all four were expressed in an *in vitro* transcription-translation system from pDS-based plasmids as described in Sections 2.14 and 2.15 and were incubated with isolated yeast mitochondria (Section 2.17). It was possible to show both the relative efficiency of binding and the efficiency of import by reisolating mitochondria from the incubation mix after allowing import to proceed for 30 minutes (Plate 4.1). It is clear that the association of the precursor with the mitochondrion is reduced with all three mutant proteins (Lanes 7,12,17) compared to wild-type β-subunit (Lane 2) as with each of the mutant proteins there is still a greater proportion of the total radioactive precursor in the supernatant fractions (Lanes 8,13,18) than in the corresponding mitochondrial fractions. Approximately 70% of the wild-type β-subunit associates with the mitochondrial fraction, but this association is most dramatically reduced when amino acids 2-35 are removed, where only approximately 30% is associated with the mitochondrial fraction, indicating that this is the region most critical for efficient targeting. Removal of either residues 2-15 or 16-35 results in a reduced association of these precursors with the mitochondria to approximately 40% and 50% respectively present in the mitochondrial fraction. It is clear that *in vitro*, both these regions are required for most efficient targeting, although some targeting still appears to take place when one or other regions are absent.

By treating the mitochondria with Proteinase K before reisolation it was also possible to show the efficiency of import of the proteins across the mitochondrial membrane (Plate 4.1). Any protein which has been imported would be protected by the mitochondrial membrane against digestion by the Proteinase K. Whereas the wild-type β-subunit precursor is matured by the matrix-located processing protease and is protected against the externally added protease (Lane 4) the mutant precursors are neither processed nor protected (Lanes 9,14,19). This indicates that removal of these amino

Plate 4.1 *In vitro* **Import of Wild Type β-subunit and Deletion Mutant Precursor Proteins to Isolated Yeast Mitochondria.**

Wild Type and mutant proteins were synthesized in an in vitro transcription/translation system in the presence of L-[^{35}S]-methionine as described in Section 2.15. Yeast mitochondria were isolated as described in Section 2.17 and 210 µg was incubated with each precursor for 30 minutes. The mixture was divided into three and incubated on ice for 15 minutes with either Proteinase K (10 µg/ml), or Proteinase K + Triton-X100 (0.3%), or nothing. The mitochondria were reisolated, and both the supernatant and mitochondrial fractions were electrophoresed using SDS-PAGE, and the gel subjected to autoradiography.

	β-SUBUNIT PRECURSOR/LANE			
FRACTION	**Wild-type**	**Δ2-15**	**Δ16-35**	**Δ2-35**
Translation	1	6	11	16
Mitochondria	2	7	12	17
Supernatant	3	8	13	18
Mitochondria + Proteinase K	4	9	14	19
Mitochondria + Proteinase K + Triton	5	10	15	20


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
β pre-
mat-

terminal residues drastically reduces the ability of the precursor proteins to be translocated across the mitochondrial membranes in this system, perhaps by reducing their correct interaction with the protein translocating machinery. It is not possible to determine from these results whether the mutant proteins are defective in being processed as no protein appears to reach the processing protease.

4.4 *In vivo* Import of Mutant Precursors.

An *in vivo* system was also used to investigate the localization of the mutant precursor proteins. This involved the expression of each of the mutant proteins from a plasmid vector within a strain of *S. cerevisiae* which no longer expressed the wild-type β-subunit. The host strain, JQ1, was constructed by inserting the *LEU2* gene into the yeast chromosome at the *ATP2* locus by one-step gene disruption, as described by Walker (1987).

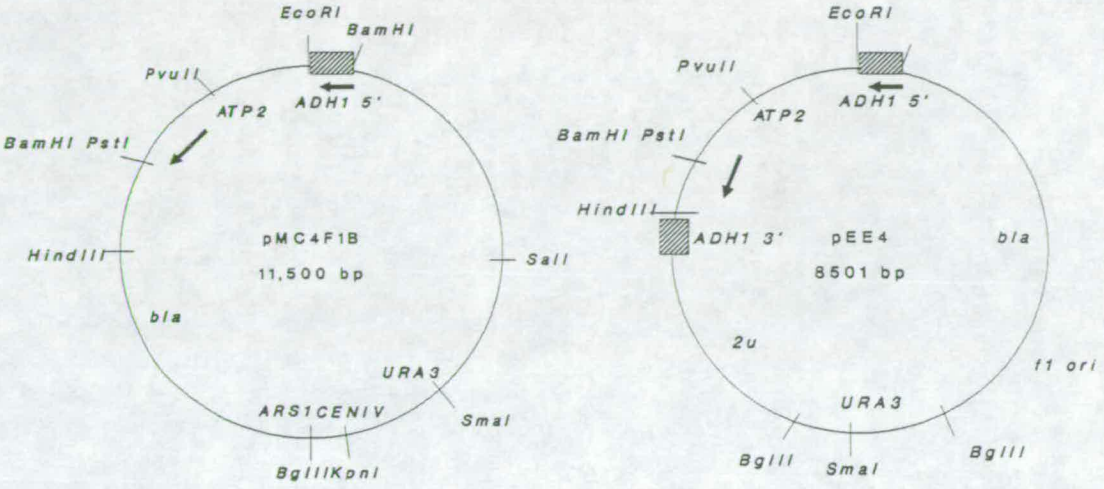
4.4.1 Expression of Mutant Precursor Proteins *In Vivo*.

In order to assess the targeting efficiency of the mutant precursors *in vivo*, strain JQ1 was transformed with plasmids carrying the appropriate *ATP2* deletions under the control of the *ADH1* promoter. The vector used, pMC4, contains an *ARS-CEN* sequence, and contains the *URA3* gene as selectable marker. The recombinant plasmids pMC4F$_1$β, pML7Y, pEE17 and pEE19 are shown in Figure 4.2, and the corresponding *S. cerevisiae* strains are MLYF$_1$β, EMY21, EMY49 and EMY50 respectively. Expression of the wild-type and mutant b-subunit from the *ADH1* promoter on these plasmids should be constitutive irrespective of the respiratory state of the cell. Levels of β-subunit protein in the cell were slightly lower than when expressed from the wild-type promoter in strain SF747 when grown on a non-fermentable carbon source such as lactate (Plate 4.2).

4.4.2 *In vivo* Complementation of the Disrupted *ATP2* Gene.

The ability to grow on a non-fermentable carbon source depends on having sufficient functional ATP synthase to synthesize ATP from the proton

Figure 4.2 Expression Plasmids of Deletion Mutants of F_1-ATPase β-subunit in Yeast.

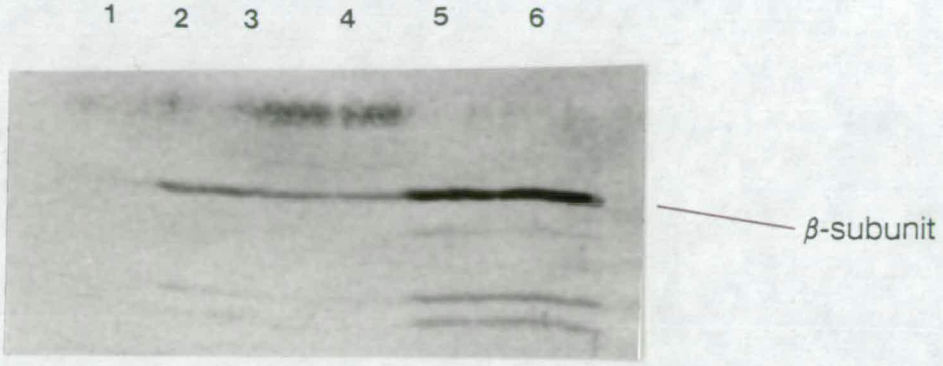


a. The wild type β-subunit gene cloned into the expression vector pMC4 on a 1.6 kb EcoRI-HindIII fragment. The deletion mutants were similarly cloned into the same vector.

b. The wild type β-subunit cloned into the expression vector pVT102A on a 1.6 kb EcoRI-HindIII fragment. The deletion mutants were similarly cloned into the same vector.

c	**Deletion**	**Plasmid**	**Yeast Strain**
Single copy	Wild Type	pMC4$F_1\beta$	MLY$F_1\beta$
(pMC4)	Δ2-15	pML7Y	EMY21
	Δ16-35	pEE17	EMY49
	Δ2-35	pEE19	EMY50
Multicopy	Wild Type	pEE4	EMY56
(pVT102A)	Δ2-15	pEE13	EMY55
	Δ16-35	pEE21	EMY51
	Δ2-35	pEE22	EMY61

Plate 4.2 Expression of β-subunit Precursor from *ATP2* and *ADH1* Promoters.



Each strain was grown shaking at 30°C in appropriate media to an OD_{600} approximately 0.7. Total cell protein was extracted as described in materials and methods, and approximately 20 μg were electrophoresed on a 10% polyacrylamide gel. Proteins were transferred by Western blotting to a nylon membrane, and probed with antisera raised against purified β-subunit.

Lane	Strain	Promoter Fusion/Plasmid	Media
1	SF747-19D	Wild-type *ATP2*	SD
2	SF747-19D	Wild-type *ATP2*	2% lactate
3	MLYF$_1\beta$	*ADH1::ATP2/ARS-CEN*	SD
4	MLYF$_1\beta$	*ADH1::ATP2/ARS-CEN*	2% lactate
5	EMY56	*ADH1::ATP2/2μ*	SD
6	EMY56	*ADH1::ATP2/2μ*	2% lactate

motive force generated by the electron transport chain. As the β-subunit is an essential component of the F_1 portion of the ATP synthase, and as the mature portion of the mutant and wild-type β-subunit precursors is identical, then any difference in ATP synthase activity in the strains harbouring the deletion mutations would be due to a difference in the amount assembling into the F_1 complex (ie reaching the mitochondrial matrix). This can be assessed by monitoring growth of the strain on a non-fermentable carbon source such as glycerol.

The strains carrying the wild type and deletion precursors were spotted out onto YPG (glycerol) plates (Plate 4.3). It is apparent that the deletion Δ2-35 results in the inability to grow on glycerol as sole carbon source, Δ2-15 results in poor growth on glycerol, and Δ16-35 results in wild-type growth on glycerol. Growth rates in liquid culture support the results from plates (Figure 4.3) and show that the rate of growth on glycerol is dramatically reduced with the deletion Δ2-35, is reduced with Δ2-15, and is similar to wild-type with Δ16-35.

These results indicate that the removal of these different regions of the presequence leads to different amounts of β-subunit assembling *in vivo*. This may be caused by a reduction in either the targeting, cleavage or assembly of the mutant precursors.

4.4.3 Overexpression of Mutant Precursors

As the amount of β-subunit expressed in the cell may have an effect on the ability of the particular protein to complement the *atp2*::*LEU2* disruption in JQ1, the wild-type and three mutant precursor proteins were also expressed from the *ADH1* promoter on a high copy number 2μ vector. The recombinant plasmids pEE4, pEE13, pEE21 and pEE22 are shown in Figure 4.2. The high copy number of 2μ based vectors (30-80 copies per cell) means that the level of expression from these vectors should be considerably higher than with the *ARS-CEN* based vectors used earlier which are only present at 2-3 copies per cell. The plasmids were transformed into JQ1 to give strains EMY56, EMY55,

Plate 4.3 Growth of Wild Type and Deletion Mutants on Glycerol Plates.

A single colony of each strain was resuspended in 100µl of distilled water, and 5µl was spotted onto the plate, and allowed to dry. Plates were incubated at 30°C for three days and growth recorded by comparison to wild type.

No	Strain	Promoter Fusion	Plasmid
1	SF747-19D	Wild type *ATP2*	-
2	JQ1	*atp2* disruption	-
3	MLYF$_1\beta$	*ADH1::ATP2*	*ARS-CEN*
4	EMY21	*ADH1*::Δ2-15*atp2*	*ARS-CEN*
5	EMY49	*ADH1*::Δ16-35*atp2*	*ARS-CEN*
6	EMY50	*ADH1*::Δ2-35*atp2*	*ARS-CEN*
7	SF747-19D	Wild-type *ATP2*	-
8	JQ1	*atp2* disruption	-
9	EMY56	*ADH1::ATP2*	2µ
10	EMY55	*ADH1*::Δ2-15*atp2*	2µ
11	EMY51	*ADH1*::Δ16-35*atp2*	2µ
12	EMY61	*ADH1*::Δ2-35*atp2*	2µ

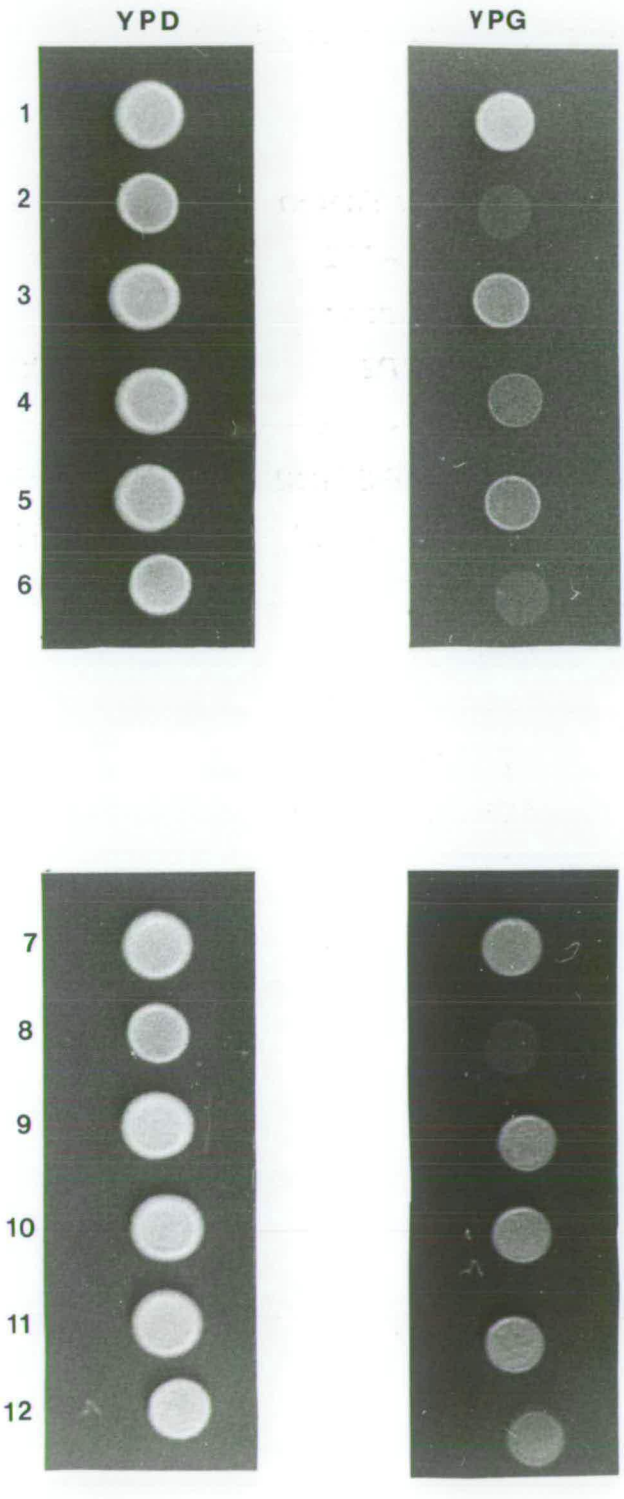
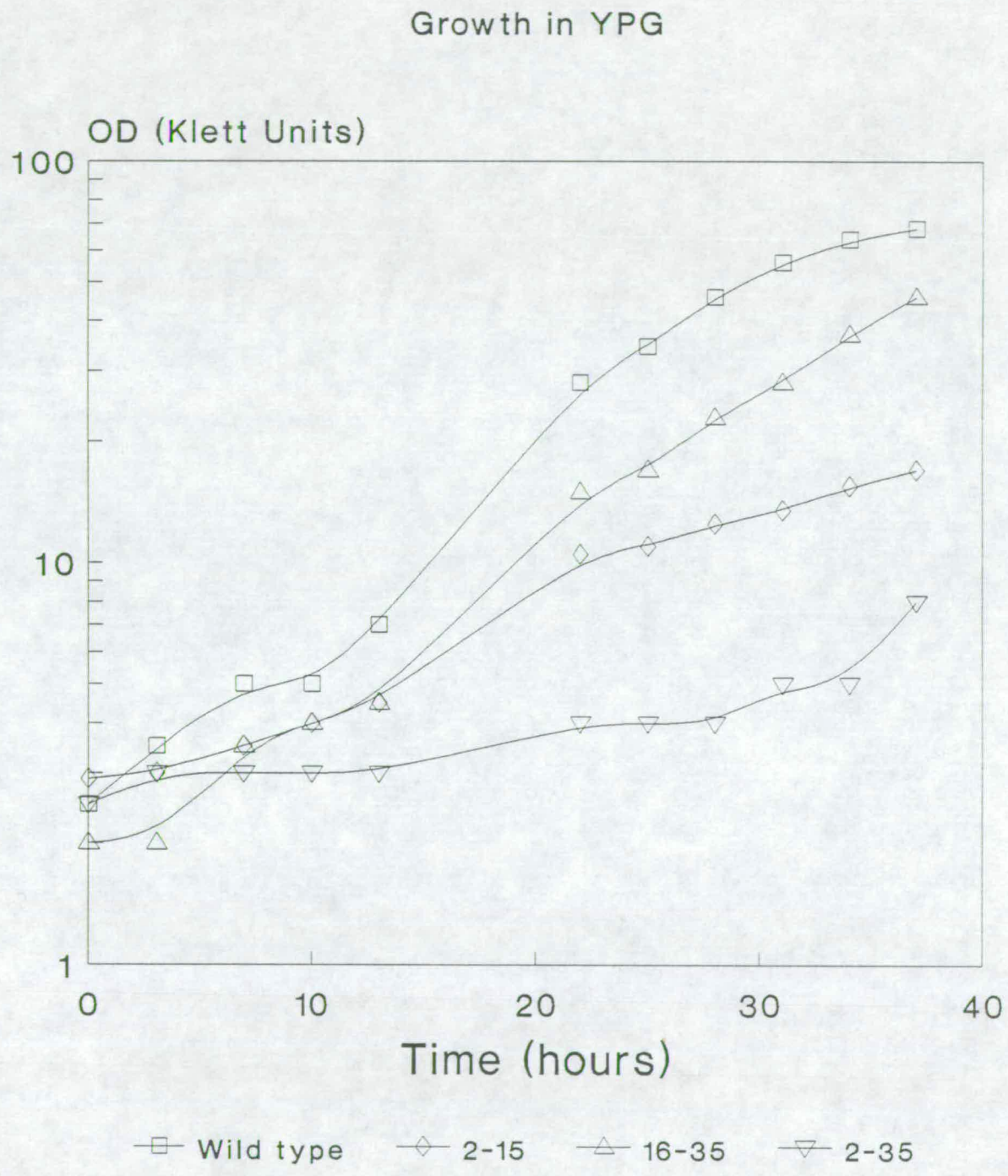

YPD
YPG
1
2
3
4
5
6
7
8
9
10
11
12

Figure 4.3 Growth in Glycerol of Strains with Wild-type and Deletion Mutant β-subunit Precursors



EMY51 and EMY61 respectively. The higher level expression of the wild-type β-subunit in strain EMY56 is clear from Plate 4.3.

The ability of the four strains to grow on glycerol as sole carbon source was determined by spotting 5μl of a resuspended colony on a YPG plate. The result (Plate 4.3) show that the deletions Δ2-15 and Δ16-35 can both complement the disruption and grow as well as the wild-type on glycerol. This is in contrast with the result obtained with the *ARS-CEN* based vector where the Δ2-15 deletion mutant did not fully complement (Figure 4.3), indicating that the phenotypic effects of deleting these residues can be overcome by increasing the pool of precursor protein.

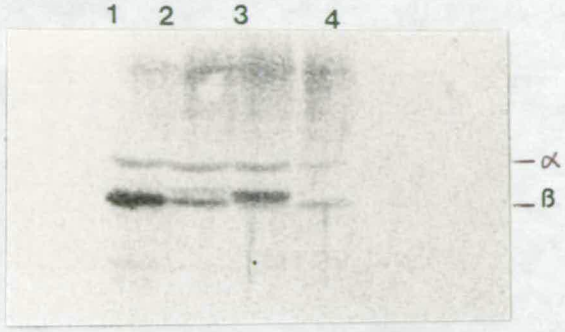
4.4.4 The Efficiency of Processing of the Mutant Precursors.

To determine the cause of the phenotypes observed on glycerol, the fate of each precursor protein in the cell was examined by extracting total cell protein from the four strains, separating by SDS-PAGE and carrying out Western blots. The results (Plate 4.4) show that approximately the same amount of β-subunit protein was expressed for each of the deletion mutants. However, with the Δ2-15 and Δ16-35 deletion polypeptides, the presence of a higher molecular weight band above the band corresponding to the mature protein indicates these precursor proteins are not being cleaved as efficiently than wild-type. The deletion Δ2-35 appears to give a protein band approximately the same size as mature, and it is difficult to ascertain from the gel whether any processing has taken place.

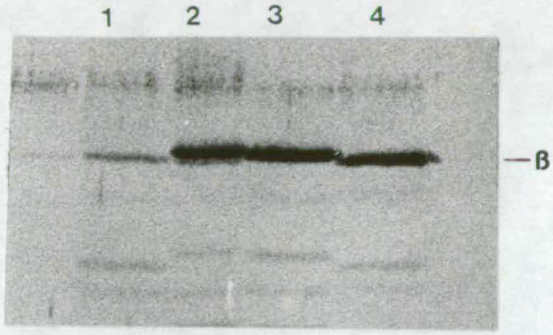
Western blot analysis of equivalent amounts of total cell protein from the strains which over express β-subunit (Plate 4.4) reveals that the level of expression is approximately 10-fold higher with the 2μ based vector. It also shows that with the Δ2-15 deletion, there is proportionately more protein remaining as the precursor form suggesting that overexpression of the mutant precursor exacerbates the defect, causing saturation of a step in the targeting pathway. In some way the cleavage of the mutant precursor is not being carried out efficiently. This could be due to either inefficient targeting,

Plate 4.4 In vivo **Processing of Wild Type and Mutant Precursor Proteins.**

a.



b.



Strains were grown to an OD_{600} of 0.7, and total cell protein was extracted as described in Section 2.11 Approximately 20 μg of protein from each sample was loaded and separated by SDS-PAGE on a 10% gel. Proteins were transferred to nylon membranes by electroblotting and probe with antisera raised against β-subunit.

Lane	Strain	Construct	Plasmid
a1	$MLYF_1b$	Wild type β-subunit	*ARS-CEN*
a2	EMY21	Δ2-15 deletion	*ARS-CEN*
a3	EMY49	Δ16-35 deletion	*ARS-CEN*
a4	EMY51	Δ2-35 deletion	*ARS-CEN*
b1	EMY56	Wild type β-subunit	2μ
b2	EMY55	Δ2-15 deletion	2μ
b3	EMY51	Δ16-35 deletion	2μ
b4	EMY61	Δ2-35 deletion	2μ

or inefficient processing of this precursor. With the Δ16-35 deletion, approximately the same amount of protein is present in the mature form as with the low level expression system, indicating that the step which is affected with this mutant is not saturated by overexpression.

4.4.5 Subcellular Localization of Mutant Precursor Proteins

In order to determine whether the mutant proteins possessed defects in the targeting or processing steps, cells from the four strains were fractionated by differential centrifugation into mitochondrial and post-mitochondrial supernatant fractions as described in Section 2.16 and the protein fractions were analyzed using SDS-PAGE and Western blots. (Plate 4.5). The results indicate that whereas almost all the wild-type protein is processed and associates with the mitochondrial fraction (Lane 3), a significant proportion of the Δ2-15 precursor is detectable in the cytoplasm (Lane 5) resulting in only a small amount of precursor, and a lower level of mature protein than wild type, being located to the mitochondria (Lane 6). Therefore it appears that the removal of amino acids 2-15 reduces the efficiency of targeting of the precursor to the mitochondria *in vivo*.

The removal of amino acids 16-35 has less of an effect on the initial stage of targeting as almost all of the protein is associated with the mitochondrial fraction (Lane 9). However, a significant amount remains as the precursor form, indicating that this deletion results in either inefficient translocation across the mitochondrial membrane; or in inefficient processing. However as the strain harbouring this deletion mutation can grow on glycerol as sole carbon source, enough Δ16-35 must still be able to assemble into a functional F_1 complex, although perhaps in an unprocessed form, and must therefore be targeted relatively efficiently.

The removal of amino acids Δ2-35 results in the majority of this protein being associated with the cytoplasmic fraction (Lane 11), and a much lower level than with the wild type associating with the mitochondria (Lane 12). This accounts for the inability of strain EMY50 to grow on glycerol, in that there is

Plate 4.5 Subcellular Localization of Wild Type and Mutant Precursor Proteins.

Cells of each strain were grown in 2% lactate media with vigorous shaking to an OD_{600} of 0.6. Mitochondria were isolated as described in Section 2.17 except that the post-mitochondrial supernatant was retained on ice. Cell equivalents of protein from each strain were separated by SDS-PAGE on a 10% gel (20 µg Total and Supernatant, 2 µg Mitochondrial). Proteins were transferred to a nylon membrane and probed with antisera against $F_1\beta$-subunit.

Lane	Strain	Precursor	Fraction
1	MLY$F_1\beta$	Wild-type	Total sphaeroplast
2	MLY$F_1\beta$	Wild-type	Postmitochondrial Supernatant
3	MLY$F_1\beta$	Wild-type	Mitochondrial
4	EMY21	Δ2-15	Total sphaeroplast
5	EMY21	Δ2-15	Postmitochondrial Supernatant
6	EMY21	Δ2-15	Mitochondrial
7	EMY49	Δ16-35	Total sphaeroplast
8	EMY49	Δ16-35	Postmitochondrial Supernatant
9	EMY49	Δ16-35	Mitochondrial
10	EMY50	Δ2-35	Total sphaeroplast
11	EMY50	Δ2-35	Postmitochondrial Supernatant
12	EMY50	Δ2-35	Mitochondrial

insufficient β-subunit assembling into the F_1 complex to allow growth on glycerol.

4.5 Determining the Cleavage Site of β-subunit.

From the above experiments, it seemed unlikely that the β-subunit was processed by the matrix protease at the published cleavage site at Lys^{19}-Gln^{20} (Vassarotti *et al.*, 1987a). As the Δ16-35 deletion overlaps this region, then as this precursor is processed to some extent, it would be necessary to assume that there was a fortuitous cleavage site in this protein if the cleavage site were correct. Processing in both the Δ2-15 and Δ16-35 deletion polypeptides gives rise to a protein which on Western blots runs in approximately the same place as mature, and is clearly distinguishable from the precursor protein. Also the Δ2-35 precursor appears to run at the same size as the processed mature protein. It was therefore estimated that processing occurs at approximately 35 amino acids into the precursor protein, to give the observed difference on Western blots when 15 amino acids were deleted from the presequence. Information obtained using N-terminal sequencing has revealed that the mature protein starts with an alanine residue, (B. Hess, personal communication) and together with the deletion data, this suggests that cleavage occurs at Ala^{34} or Ala^{36} (Figure 4.1).

From the deduced amino acid sequence of the β-subunit protein, it is apparent that there is only one cysteine residue in the entire β-subunit precursor protein at residue 32 (Figure 4.1), and this lies between the published cleavage site at 19-20 and the one proposed at 34 or 36. To determine the site of processing, radioactive wild-type precursor was synthesized using reticulocyte lysate in the presence of either [^{35}S]-methionine, or [^{35}S]-cysteine, and was incubated with isolated yeast mitochondria (Plate 4.6). In both cases the precursor form is present, but the protein which is labelled with [^{35}S]-cysteine is not detectable after processing by the mitochondrial matrix protease, leaving no labelled protein as the mature form. Presumably, the stretch of amino acids containing the cysteine

Plate 4.6 Cleavage of Radioactive Precursors of F_1-ATPase β-subunit *in vitro.*

Radioactive precursor was synthesized in a transcription/translation system as described in Section 2.16 using either [^{35}S]-methionine or [^{35}S]-cysteine. Each precursor was incubated with isolated yeast mitochondria for 30 minutes, and the mitochondria were reisolated from the mix. Samples were analyzed using SDS-PAGE and flurography.

Lane	Label	Fraction
1	[^{35}S]-methionine	Total translation product
2	[^{35}S]-methionine	Mitochondrial fraction
3	[^{35}S]-methionine	Post-mitochondrial supernatant
4	[^{35}S]-cysteine	Total translation product
5	[^{35}S]-cysteine	Mitochondrial fraction
6	[^{35}S]-cysteine	Post-mitochondrial supernatant

has been cleaved off by the protease to a small peptide which is no longer visible on the gel due to its small size. This provides evidence that the precursor protein is cleaved after the cysteine residue at 32, suggesting that the cleavage site is not at Lys^{19}-Gln^{20} but more probably at Ala^{34} or Ala^{36}.

4.6 Discussion

In this Chapter, the effects of deleting amino acid residues from the amino terminus of the β-subunit of the F_1 ATPase on the targeting of the protein to the mitochondrial matrix and on its subsequent processing have been described. It is apparent that the removal of the first 35 amino acids (Δ2-35) severely impairs the targeting process, both *in vitro* and *in vivo*. This indicates that these residues perhaps interact in some way with the protein translocating machinery to achieve efficient targeting. Obviously, the prerequisite of efficient binding will influence the ability of a protein to be translocated, and with the Δ2-35 deletion the inefficiency of the initial targeting step seen *in vitro* may have a dominating influence on the subsequent translocation step(s).

The deletion of residues 2-15 affects the efficiency of processing of this protein both *in vitro* and *in vivo*. In both cases, a substantial amount of protein fails to be imported into the mitochondria. Therefore the inability of the Δ2-15 deletion protein to fully complement the *atp2* disruption can be accounted for by its failure to be targeted efficiently. From the localization experiments, it appears that the step which is affected is the initial targeting step and this results in the accumulation of precursor in the cytoplasm, seen to a greater extent when the protein is overexpressed using a 2μ vector. *In vivo*, the unprocessed form can still be detected in the cytoplasm under steady-state conditions, and it appears that some degradation of this protein occurs, resulting in less precursor protein being present than would be anticipated. This phenomenon suggests either that targeting occurs rapidly enough with the wild-type precursor to prevent degradation; or that the cell

can in some way recognize the mutant protein with its partial signal sequence and degrade it preferentially.

The deletion Δ16-35 still complements the *atp2* disruption but clearly displays a deficiency in processing *in vivo*, and a deficiency in targeting *in vitro*. Sufficient precursor is assembling to give functional ATPase, even though it is not processed fully. It has been proposed that one function of a presequence is to have an effect on precursor structure during the targeting/translocation process, to help maintain it in an import-competent state and to prevent it from assembling together with other subunits before it reaches its destination. However if this is the case, then it appears that this deleted presequence is not affecting the precursor structure to a significant extent once it has reached the mitochondrial matrix as it can still assemble into a functional complex. This may be because possible accessory proteins which prevent the precursor form folding in the cytoplasm such as chaperonins are not present within the mitochondria; or it is possible that the deletion has reduced the ability of the presequence to exert an effect on the precursor structure - this could account for the reduced targeting ability seen *in vitro*.

The observation that sufficient targeting occurs *in vivo* to allow complementation illustrates the problems of relying solely an *in vitro* system as an assay for targeting efficiency. However, the results *in vitro* may be highlighting a targeting defect which is too subtle to be detected at steady state levels in an *in vivo* system. The requirement of both the 2-15 and 16-35 parts of the presequence for 100% efficiency of targeting suggests that it is possible that these two regions act in an additive manner.

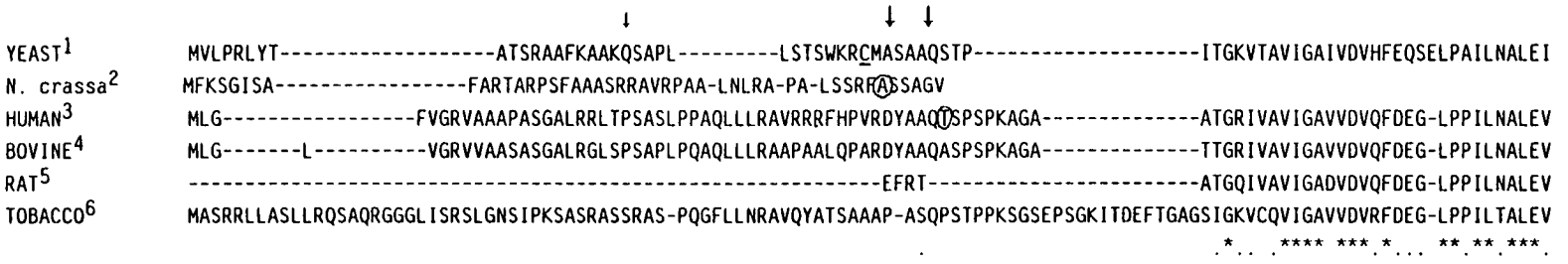
The work of Bedwell *et al.* (1987) correlates with these findings that regions of the β-subunit presequence may be acting synergistically in the targeting process in that the efficiency of import is affected by the deletion of different regions of the presequence. They also define the first 35 amino acids as being absolutely essential for efficient targeting. However, the failure

to positively identify an incomplete complementation of the *atp2* gene with a very similar Δ2-19 deletion may be due to the fact that the wild-type *ATP2* promoter was used as opposed to the *ADH1* promoter used in this study. As more protein is expressed with the *ATP2* promoter when the cells are grown on glycerol, it appears that the use of the *ADH1* promoter gives a more sensitive assay for targeting efficiency *in vivo*. Other groups have similarly found that removal of some regions of the presequence results in partial targeting capability of the mutant protein. The critical region which is absolutely required for targeting of pre-ornithine transcarbamylase to rat liver mitochondria was shown to be residues 8-22, but deletion polypeptides Δ2-7 and Δ2-12 gave a reduced level of targeting *in vitro* (Horwich *at al.,* 1986).

Sufficient of the Δ16-35 precursor is assembling to give functional ATP synthase even though it is not processed fully. This leads to the conclusion that even with a shortened presequence attached the β-subunit can still fold into a functionally compatible conformation. This phenomenon was also observed by Vassarotti *et al.* (1987) who observed the ability of deleted precursors to assemble into the F_1 ATPase. Some mitochondrial precursors do not appear to be cleaved upon reaching the mitochondrial matrix, but are still able to assemble (Hampsey *et al.,* 1983). However the cleavage of other precursors must be essential, as a deficiency in processing activity (such as occurs in a strain carrying the *mas1* mutation) is lethal. It seems that the requirement for cleavage varies depending on the polypeptide.

The reduced extent of processing observed with the Δ16-35 deletion suggests that the recognition site for the processing protease has been partially deleted. This supports results obtained by Vassarotti *et al.,* (1987), who showed that small deletion polypeptides 17 residues from their proposed processing site at Lys^{19} (that is, around residue 36) prevented protease-dependent removal of the presequence; and also that a larger deletion Δ16-37 failed to be processed either *in vivo* or *in vitro*. The structure which is recognized by the processing protease is unknown, although Hurt *et*

Figure 4.4 Comparison of Presequences of Mitochondrial F_1 β-Subunits from Different Species



Sequences were aligned using CLUSTAL.

KEY

* :=> match across all seqs.

. :=> conservative substitutions

↓ - possible cleavage site in yeast β-subunit.

1. Takeda *et al.*, 1985.
2. Rassow and Neupert, unpublished.
3. Ohta *et al.*, 1988.
4. Breen *et al.*, 1988.
5. Garboczi *et al.*, 1988.
6. Boutry and Chua, 1983.

Cysteine residue in yeast β–subunit is underlined

Circled amino acids indicate the start of mature sequence, where known.

al.,(1987) propose that it is formed from several parts of the presequence, not necessarily around the known cleavage site.

It is also clear that the cleavage site is not at Lys^{19}-Gln^{20} as previously reported (Vassarotti *et al.,* 1987) but is more probably located at Ala^{34}. The fact that other β-subunit proteins commence at a similar point and that this region is more conserved across species than the targeting sequence supports this result (Figure 4.4). As the Δ2-35 deletion cannot complement the *atp2* disruption even when overexpressed, it seems that the cleavable presequence (residues 1-35) contains most of the information for efficient interaction of the precursor protein with components of the protein-localization machinery.

CHAPTER FIVE

ISOLATION AND CHARACTERIZATION OF MUTANTS WHICH SUPPRESS A DEFECTIVE MITOCHONDRIAL TARGETING SEQUENCE

5.1. INTRODUCTION

It is now well established that protein factors are involved at various steps in the localization of mitochondrial precursors (Chapter 1). For example, the existence of cytoplasmically-located targeting recognition proteins has been proposed (Bernstein *et al.,* 1989; Section 1.3.1). Components of a receptor complex on the mitochondrial outer memebrane are thought to interact specifically with the presequence (Zwizinski *et al.,* 1984; Riezman *et al.,* 1983; Section 1.3.2). Several proteins have been implicated recently in keeping the precursor protein in a transport-competent state, such as the HSP70 proteins (Deshaies *et al.,* 1988) and may even cause unfolding of the precursor Section 1.4.1.1). Proteins responsible for cleaving the presequence to yield the mature form of the protein (matrix-located protease) have been identified also (McAda and Douglas, 1982; Section 1.5). Proteins such as Hsp60 are thought to be involved in the sorting and assembly of mitochondrial subunits (Cheng *et al,* 1989; Section 1.7).

Some of these protein components such as the MOM19 receptor on the mitochondrial outer surface (Sollner *et al.,* 1989) have been isolated by biochemical means, whereas others such as the matrix-located processing peptidases have been isolated by genetic means (Yaffe and Schatz, 1984). However reconstitution *in vitro* of the many components involved has been thwarted by lack of knowledge of the number of components and the absence of an assay for their specific roles.

In order to identify proteins involved in mitochondrial protein transport it is possible to take full advantage of the genetics available with yeast. The use of yeast genetics has led to the identification of several genes which are involved in the secretory pathway (Novick *et al.,* 1980). Mutants of other genetically amenable micro-organisms such as *E. coli* have been used to identify components of their protein-targeting machinery, for example the SecA, SecB and SecY gene products (reviewed in Randall *et al.,* 1987). For mitochondrial protein targeting, only three groups of components have been

identified genetically - a cytosolic chaperone, the matrix peptidase and a mitochondrial chaperone. The genetic approaches used to identify these components are discussed below.

The prediction of the involvement of ATP-dependent heat shock proteins in the targeting process led to the implication of the *SSA1, SSA2, SSA3* and *SSA4* genes, which were already known to encode hsp70 proteins. A yeast mutant which was defective in all four genes accumulated precursor of the β-subunit (Deshaies *et al.,* 1988).

Several selection schemes have been devised to isolate new mutants in targeting components. One approach involved screening a pool of temperature-sensitive yeast mutants for the loss of ability to grow on glycerol, and then for the accumulation of precursor forms of mitochondrial proteins, using western blot analysis (Yaffe and Schatz, 1984). This yielded the mutants *mas1* and *mas2* and these were later shown to encode two components of the matrix protease MPP and PEP (Witte *et al.,* 1988; Jensen and Yaffe, 1988). One drawback to this approach is that there are many mutations which can lead to the loss of ability to grow on glycerol, some of which affect the mature part of proteins required for oxidative phosphorylation and others the presequences of such proteins.

Another approach used a more elaborate screen in which the precursor form of human ornithine transcarbamylase (OTC) was fused to the *GAL1* promoter (Pollock *et al.,* 1988). By screening temperature sensitive lethal mutants for the loss of OTC activity upon temperature upshift and simultaneous induction of expression from the *GAL1* promoter, the mutations *mif1, mif2* and *mif3* were isolated. However, two of these mutations, *mif1* and *mif2*, were allelic to *mas1* and *mas2*, and were shown to encode the matrix-located protease components MPP and PEP (Pollock *et al.,* 1988). The third mutation *mif3* is as yet uncharacterised. This screen also yielded the mutant *mif4* which encodes an Hsp60 protein, and which is involved in the sorting and assembly of proteins in the mitochondrial matrix. As yet, no yeast mutant

has been isolated which is defective in the recognition and binding of a precursor to the mitochondrial outer membrane or that is defective in translocation.

An alternative approach to those described above would be to isolate extragenic mutations which suppress a known defect in a mitochondrial presequence. The principle of extragenic suppression is outlined in Figure 5.1. The feasibility of this approach has already been shown in another system, that of protein export in *Escherichia coli* (Emr *et al.,* 1983). Using a deletion in the signal sequence of the LamB protein, extragenic suppressor mutations were isolated which restored the ability of the mutant LamB protein to be exported. Genetic and biochemical characterization of one of these mutations showed that it lay in a gene encoding a component of the protein translocating pathway, *secY*. This protein is now known to be an integral membrane protein of the *E. coli* plasma membrane. The advantage of this strategy is that it is specific for genes whose products interact directly with the presequence, and avoids screening unrelated defects. This method relies on an identifiable phenotype on plates, such as that observed with the Δ2-15 deletion in the presequence of β-subunit (Section 4.4.2; Plate 4.3).

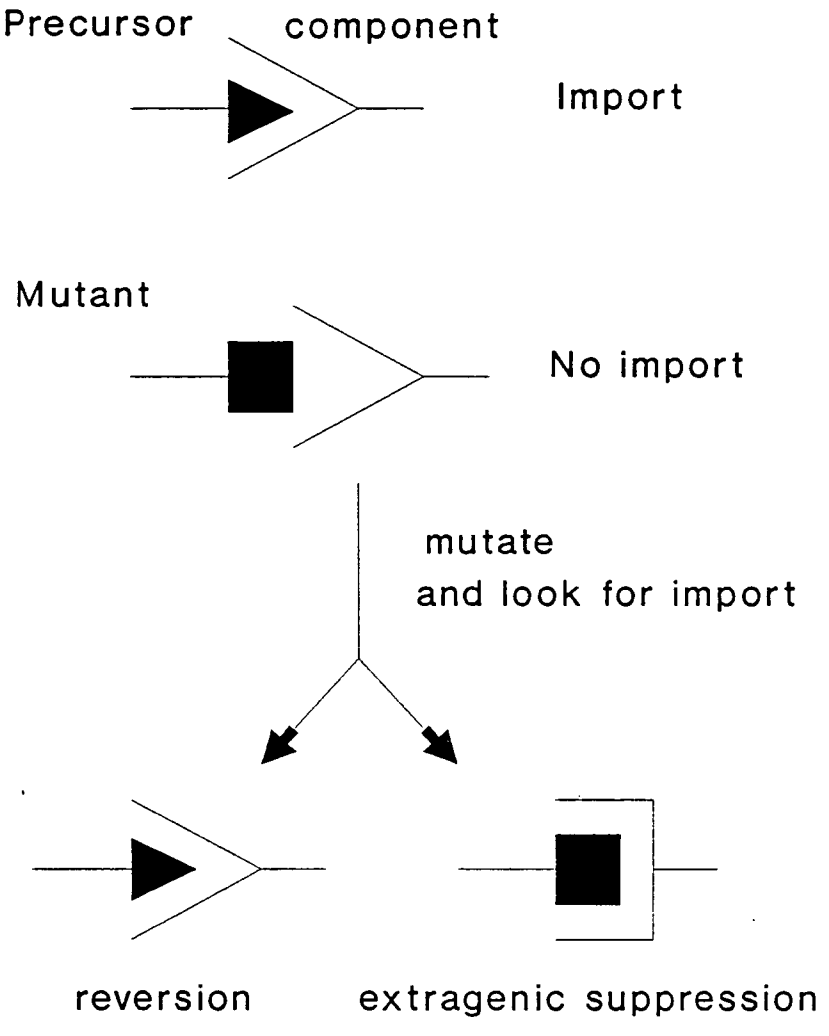
As it is possible that a similar mechanism of recognition may exist for mitochondrial targeting as for bacterial secretion, the isolation of mutants which were able to suppress the defective mitochondrial targeting sequence Δ2-15 was attempted.

5.2 MUTANT ISOLATION

5.2.1 Mutant Isolation Strategy

The defective mitochondrial targeting sequence Δ2-15 leads to the strain EMY21 showing reduced growth on glycerol as sole carbon source (Plate 4.3). It was hoped that mutants of EMY21 which showed improved growth on glycerol would be able to suppress the original growth defect by enabling more β-subunit to be localized to the mitochondria. This increase in targeting

Figure 5.1 Principle of Extragenic Suppression



could be caused by a mutation altering an import component to increase its affinity for the modified β-subunit. Such mutations may also cause the component to be unstable at a higher temperature and therefore render it non-functional. As it is known that some degree of mitochondrial function is absolutely required for the synthesis of essential metabolites such as sterols (Henry *et al.,* 1982), even when the cell is grown in the presence of glucose (Kovacova, 1968), the total abolition of all import processes may be fatal for the cell. Therefore, a mutation in a component common or essential to import may give rise to a temperature-sensitive phenotype. Such a phenotype would simplify subsequent biochemical and genetic analysis, and would also allow the rapid cloning of the wild type gene. For this reason, the original screening for suppressors was carried out at 25°C, and the cells were then screened for their inability to grow at 35°C.

5.2.2 Isolation of Temperature-Sensitive Suppressors

Mutants of the yeast strain EMY21 were isolated after subjecting a stationary phase culture to UV mutagenesis as described in Section 2.18.2.The number of cells screened was determined using serial dilutions of the mutagenized cultures on YPD plates and incubating at 25°C for 2 days. Mutants colonies were screened for their ability to grow on glycerol plates containing limiting glucose (SDG) (Section 2.3.2) to a greater extent than the original parental strain EMY21 after incubating the plates at 25°C for six days. The screen for temperature sensitive mutants was carried out on SDG plates, as a temperature sensitive phenotype may be more apparent if it blocked oxidative phosphorylation. Colonies from the original screen were streaked onto a fresh SDG plate, replica plated to another SDG plate and incubated at either 25°C or 35°C. The isolation of mutants is outlined in Table 5.1.

Two mutants were isolated by this screen and were designated EMY172 and EMY153. Their phenotypes on glycerol and glucose media are shown on Plates 5.1 and 5.2. This clearly shows that both strains suppress the original defect in EMY21. However whereas EMY172 is extremely sensitive to an

Table 5.1 Isolation of Suppressor Mutants of Δ2-15 Deletion of pre-$F_1\beta$-subunit.

STEP	RESULT
UV Dose	2 minutes at 10 ergs/mm^2/sec
% Killing	50%
No. Cells Screened	2×10^8
No. Positives (glycerol$^+$)	246
Frequency glycerol$^+$	1.23×10^{-6}
No. Temperature Sensitive	2
Frequency ts glycerol$^+$	1×10^{-8}

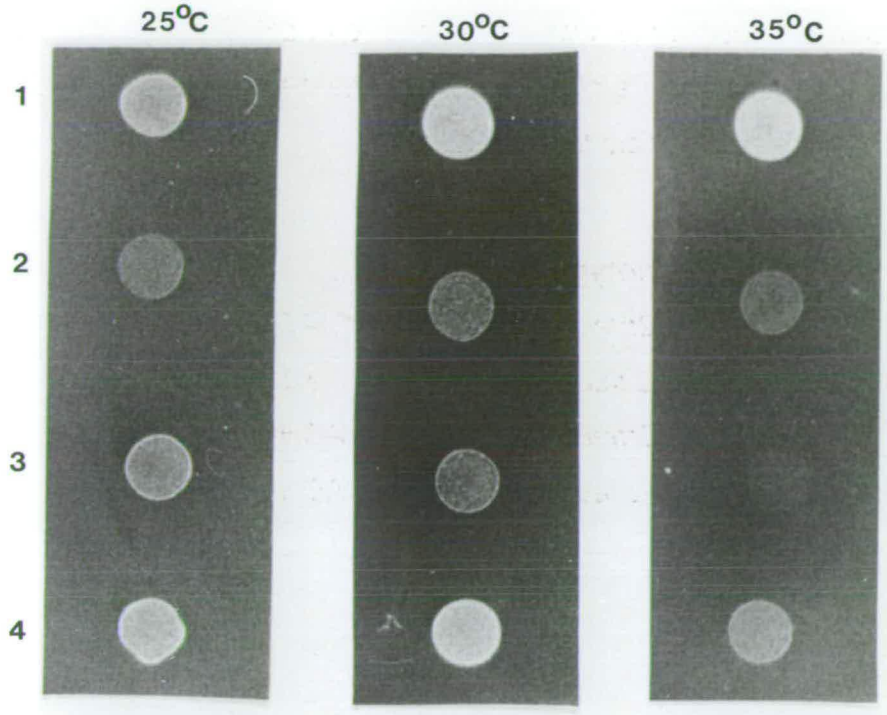
Yeast were mutagenized as described in Section 2.2, allowed to grow in the dark for 2 days, and plated out on the appropriate media. The initial selection was carried out by growing on SDG (limiting glucose) plates for six days, and the screen for temperature sensitivitity was carried out on YPD and YPG plates.

Plate 5.1 Phenotypes of Suppressor Mutants on Glycerol Plates

A single colony of each strain was resuspended in dH_2O and 5 µl of each was spotted onto the appropriate plates. Growth was photgraphed after three days incubation at the appropriate temperature.

No.	Strain	Genotype
1	MLYF$_1\beta$	*atp2::leu2* /p*URA3 ADH1::ATP2*
2	EMY21	*atp2::leu2* /p*URA3 ADH1::*Δ2-15*atp2*
3	EMY172	*atp2::leu2 mts1* /p*URA3 ADH1::*Δ2-15*atp2*
4	EMY153	*atp2::leu2 mts2* /p*URA3 ADH1*::Δ2-15*atp2*

YPG



SDG

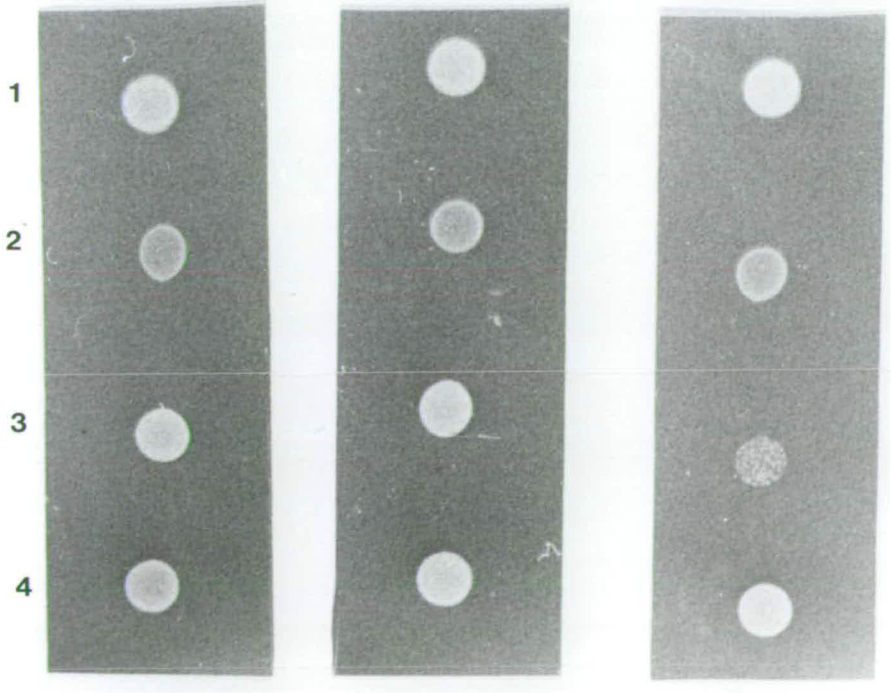
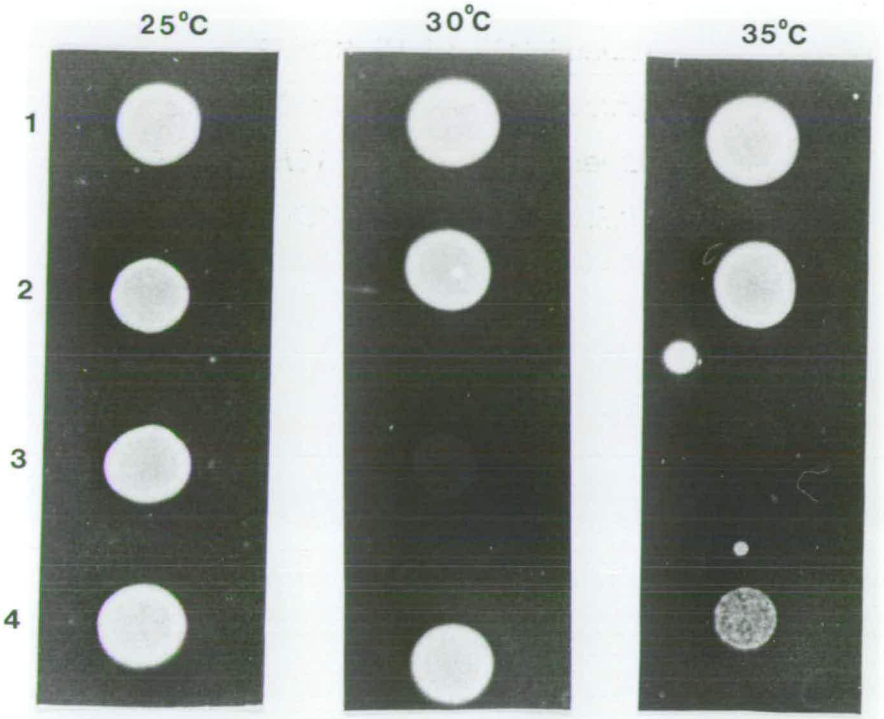


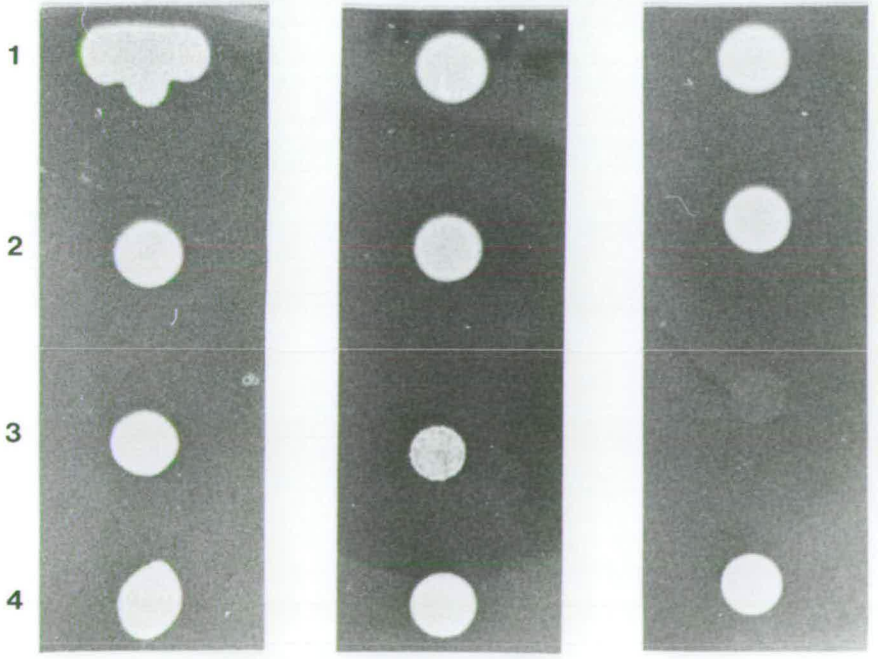
Plate 5.2 Phenotypes of Suppressor Mutants on Glucose Plates

No.	Strain	Genotype
1	MLYF$_1\beta$	*atp2::leu2* /p*URA3 ADH1::ATP2*
2	EMY21	*atp2::leu2* /p*URA3 ADH1::*Δ2-15*atp2*
3	EMY172	*atp2::leu2 mts1* /p*URA3 ADH1::*Δ2–15*atp2*
4	EMY153	*atp2::leu2 mts2* /p*URA3 ADH1*::Δ2-15*atp2*

YPD



SD



increased temperature, EMY153 is not quite so temperature sensitive on glucose and not at all temperature sensitive on glycerol. With both mutants it is apparent that the temperature-sensitivity was greater on glucose plates than on glycerol plates. The reason for this is not clear.

5.3 GENETIC CHARACTERIZATION OF SUPPRESSOR MUTANTS

5.3.1 Suppression is Not Plasmid-Borne

It was possible that the suppressor mutants isolated were not defective in an import component but may be intragenic pseudo-revertants of the original deletion in the presequence of the β-subunit. For example, a point mutation resulting in an amino acid change in a mutant presequence gave rise to a functional targeting sequence (Vassaroti *et al.* 1986). It is possible to distinguish between these alternatives by isolating plasmid carrying the Δ2-15 deletion from the suppressor strains, and transforming them back into the original *ATP2* disruption strain JQ1. A mutation leading to an alteration in the presequence would be plasmid-borne, whereas a true extragenic suppressor mutation would reside in a chromosomal or mitochondrial gene.

Plasmid DNA from strains EMY172, EMY153 and EMY21 was isolated as described in Section 2.6.3.1, and was used to transform *E. coli* strain MM294 to Amp^r. The three plasmids recovered from each strain (p172 p153, and p21 respectively) were propagated in *E. coli*, and used to transform the yeast strain JQ1 to URA^+ at 25°C. Transformants were picked and tested for their ability to suppress the Δ2-15 deletion by spotting them on YPG (glycerol) plates, and also for their temperature sensitivity by spotting on YPD (glucose) plates at 35°C. The results are shown in Table 5.2a and show that neither the plasmid from strain EMY172 nor the plasmid from strain EMY153 confer the ability to suppress the 2-15 deletion in the β-subunit presequence, and that it is likely that both mutations are either in a chromosomal or mitochondrial gene. Also the temperature sensitivity does not appear to be carried on the plasmid.

Table 5.2 Suppression is Not Plasmid-borne.

a. Plasmids Isolated from Suppressor Mutants do not Confer the Ability to Suppress.

			Growth			
			YPD		YPG	
Strain	Rel. Genotype	Plasmid	25°C	35°C	25°C	35°C
EMY21	Wild type	(pML7Y)	+++	+++	+	+
EMY172	*mts1*	(pML7Y)	+++	-	+++	+
EMY153	*mts2*	(pML7Y)	+++	+	+++	++
JQ1	Wild type	none	++	++	-	-
JQ1	Wild type	+p21	+++	+++	+	+
JQ1	Wild type	+p172	+++	+++	+	+
JQ1	Wild type	+p153	+++	+++	+	+

b. Cured Suppressor Mutant is Able to Suppress when Retransformed.

			Growth			
			YPD		YPG	
Strain	Rel Genotype	Plasmid	25°C	35°C	25°C	35°C
EMY54	*mts1*	-	+++	-	-	-
EMY54	*mts1*	pML7Y	+++	-	+++	+
EMY172	*mts1*	(pML7Y)	+++	-	+++	+
EMY21	*mts1*	(pML7Y)	+++	+++	+	+

Strains were tested by resuspending a single colony in 100 µl sterile water in a microcentrifuge tube, and spotting 5 µl onto the appropriate plate. Growth was recorded after 3 days incubation.

To further verify that the mutation was not plasmid-borne, strain EMY172 was cured of its plasmid by growing in non-selective media (YPD) to stationary phase and plating dilutions on YPD. From this plate, 200 colonies were picked onto SD+H plates and SD+H+U plates. Out of these, 3 had become URA^-. One of these (EMY54) was retransformed to URA^+ with plasmid pML7Y carrying the Δ2-15 deletion in the β-subunit presequence. The resultant strain and the parental strain, were spotted on to YPD and YPG plates and grown at 25°C or 35°C for 3 days. The results in Table 5.2.b show that the cured strain is temperature-sensitive, and that the retransformed strain is still able to suppress the targeting defect as well as being temperature-sensitive, thus confirming that suppression is not plasmid-borne.

5.3.2 The Suppressor Mutation is a Temperature Sensitive Mutation in a Nuclear Gene.

Assuming that the mutations in EMY172 and EMY153 lay in essential components of the targeting machinery, it would be unlikely that they were mitochondrially-encoded, because strains with no mitochondrial DNA (ρ^o) have been reported to be able to import mitochondrial precursors (Tzagaloff, 1986). However to confirm that the mutations causing suppression of the targeting defect were in nuclear genes, and to test whether the ability to suppress and the temperature-sensitive phenotype were linked, tetrad analysis was carried out after mating the suppressor strain with an equivalent wild-type strain of the opposite mating type. It was decided to proceed with only one strain, EMY172, as the temperature sensitivity of strain EMY153 was sometimes difficult to distinguish.

Strain DBY747 (MAT a, *MTS1*) was mated to EMY172 (MAT α, *mts1)* to give the diploid EMY40. Strain EMY40 was allowed to sporulate, and the asci dissected as described in Section 2.18.3. Spores were germinated on YPD plates at 25°C. and their phenotypes were examined by spotting out onto the appropriate plates at 25°C and 35°C. The results in Table 5.3 clearly show that the temperature sensitive phenotype segregates in a Mendelian pattern

Table 5.3 *mts1* **is a Temperature Sensitive Mutation in a Nuclear Gene.**

DBY747 a *his3-Δ1 leu2-3 leu2-112 ura3-52 trp1*

x

EMY172 α *his4 leu2-3 leu2-112 ura3-52 mts1 atp2::LEU2 /pURA3 ADH1::Δ2-15atp2*

EMY40 *his3-Δ1 HIS4 leu2-3 leu2-112 ura3-52 trp1 MTS1 ATP2*
a/α *HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 mts1 atp2::LEU2 /pURA3 ADH1::Δ2-15atp2*

a. Temperature sensitivity segregates in a Mendelian pattern

Tetrad	Spore	25°C	35°C	Tetrad	Spore	25°C	35°C
1	a	+	+	6	a	+	-
	b	+	+		b	+	+
	c	+	-		c	+	-
	d	-	-		d	+	+
2	a	+	-	7	a	+	+
	b	+	-		b	+	-
	c	+	+		c	-	-
	d	-	-		d	+	+
3	a	-	-	8	a	+	+
	b	+	-		b	+	-
	c	+	+		c	+	-
	d	+	+		d	+	+
4	a	+	+	9	a	+	-
	b	+	-		b	+	+
	c	-	-		c	-	-
	d	+	+		d	-	-
5	a	+	+	10	a	+	+
	b	-	-		b	+	+
	c	-	-		c	+	-
	d	+	-		d	-	-

b. Suppression is linked to temperature sensitivity

Phenotype at 25°C	No Progeny Tested	Growth at 35°C
URA$^+$LEU$^+$GLY$^+$	53	0
URA$^+$LEU$^+$GLY$^-$	67	66

The strain EMY40 was induced to sporulate and tetrads were dissected using a micromanipulator. All progeny were tested for their phenotypes by spotting on various plates. As the ability to suppress the Δ2-15 deletion is only apparent in strains which have the wild-type ATP2 gene disrupted (ie LEU$^+$) and which possess the plasmid carrying the deletion construct (ie URA$^+$), only progeny which were LEU$^+$, URA$^+$ were screened for the linkage of the suppression phenotype to the temperature sensitive phenotype.

thus confirming that the lesion causing the temperature sensitivity is nuclear-encoded. The results also show that suppression is genetically-linked to the temperature sensitive phenotype in all the spores examined which were LEU^+ (ie $ATP2^-$) and URA^+ (ie possessed the plasmid with Δ2-15*atp2*). Therefore because of the low frequency of mutation from the original mutagenesis (Table 5.1), it is likely that the mutation causing suppression also causes the temperature sensitive phenotype. The mutation was named *mts1* (for **m**itochondrial **t**argeting **s**uppressor).

5.3.3 Inability of *mts1* to Suppress Δ2-35 Deletion in the Presequence of β-subunit

It was possible that the suppressor was acting by increasing the affinity of the Δ2-15 deleted β-subunit precursor with a component of the mitochondrial import machinery. It was important to determine whether the *mts1* mutation could also affect the efficiency of targeting of the Δ2-35 deleted β-subunit protein. A true extragenic suppressor might be expected to be allele-specific, and would be unable to suppress a different mutation in the β-subunit presequence. A more general suppressor may be able to affect the import of other mutants. Also, as the 2-35 deletion no cleavable presequence, an increase in its import in the suppressor background might suggest the involvement of the mature part of the β-subunit in its binding to import components. Strain EMY53 (the suppressor strain cured of the plasmid pML7Y, Section 5.3.1) was transformed with plasmid pEE19 (carrying the Δ2-35 deletion). The resultant strain was spotted out on a YPG plates at 25°C with appropriate controls. The results are shown in Table 5.4. This clearly demonstrates that the suppressor *mts1* is unable to suppress the deletion Δ2-35 and this suggests that the mature part of the β-subunit is not implicated in the proposed increased interaction between the putative import component and the β-subunit precursor, and therefore that the majority of essential targeting information resides in the cleavable presequence of the β-subunit.

Table 5.4 *mts1* **is Unable to Suppress Δ2-35 Precursor Defect.**

Strain	Plasmid	Relevant Genotype	Glycerol	Glucose
MLYF$_1\beta$	pMC4F$_1\beta$	*ATP2*	+ + +	+ + +
EMY172	pML7Y	*Δ2-15atp2, mts1*	+ + +	+ + +
EMY54	-	*atp2::LEU2, mts1*	-	+ + +
EMY59	pEE19	*Δ2-35atp2, mts1*	-	+ + +
EMY50	pEE19	*Δ2-35atp2*	-	+ + +

EMY172 was cured of its plasmid to give strain EMY54, and this was retransformed with plasmid pEE19 carrying the Δ2-35 deletion to give strain EMY59. Strains were spotted onto the appropriate plates and grown for 3 days at 25°C.

5.4 Possible Mechanisms of Suppression

Because of the selection scheme adopted to isolate the *mts1* mutation , it was hoped that suppression of the Δ2-15 deletion was caused by increasing the affinity of an import component for the deleted precursor. However it was also possible that the *mts1* gene did not encode an import component, but was allowing suppression by a secondary effect, either by affecting glycerol utilization, or by increasing the expression of the deleted precursor polypeptide.

5.4.1 Glycerol Utilization

The *mts1* mutation may be causing increased growth on glycerol by affecting glycerol utilization. To eliminate this possibility, cells were spotted out on YPL (lactate medium). The results in Table 5.5 shows that the suppressor phenotype caused by *mts1* is also observed when the strain EMY172 is using lactate as sole carbon source. As lactate is utilized by a separate pathway, then it is unlikely that the suppression observed is due to an alteration in glycerol utilization.

To rule out the possibility that indirect effects were influencing the ability to grow on glycerol, the activity of ATPase was measured in mitochondria isolated from the mutant and wild-type cells. The results are shown in Table 5.6, and show an increase in ATPase acitivity in EMY172 compared to the activity in the strain with the 2-15 deletion (EMY21). However activity is not as high as wild type levels of $MLYF_1$ β. This assay correlates well with the phenotype shown on plates, (Plate 5.1) and confirms that the improved growth on glycerol is due to an increase in ATPase acitvity. Such an increase could arise by improved targeting efficiency of β-subunit to the mitochondria.

5.4.2 Increased Expression of the Δ2-15 Precursor.

As an initial screen for the effects of *mts1* on mitochondrial protein import, cell extracts were analyzed by Western blots to ascertain whether the amount of deleted precursor in the cytoplasm was reduced, and if the

Table 5.5 Phenotype of Suppressor Strain on Lactate media.

Strain	Relevant Genotype	YPG	YPLactate
MLYF$_1\beta$	*ATP2*	+ + +	+ + +
JQ1	*atp2::LEU2*	-	-
EMY21	*Δ2-15atp2*	+	+
EMY172	*Δ2-15atp2, mts1*	+ + +	+ + +

Strains were spotted on the appropriate plates and grown at 25°C for 3 days.

Table 5.6 Mitochondrial ATPase Activity in Mutant and Wild-Type Strains

Strain	Relevant Genotype	ATPase Activity μmol/min/mg	% Wild Type
MLYF$_1\beta$	*ATP2*	1.56±0.08	100
EMY21	*Δ2-15atp2*	0.89±0.03	57
EMY172	*Δ2-15atp2, mts1*	1.21±0.06	77

Strains were grown in 2% Lactate media at 25°C to an OD_{600} of 0.6 before harvesting. Mitochondria were isolated from each strain as described in section 2.16, and ATPase activity was assayed as described in Section 2.19. The assay was carried out in triplicate for each strain.

amount targeted to the mitochondria was increased. This would also show whether or not the suppressor is acting by increasing the expression of β-subunit from the *ADH* promoter and is therefore mimicking the effects found with the deletion expressed from the multicopy vector as shown in Plate 4.3.

Subcellular fractionations were prepared as described in Section 2.16, of strains EMY21 and EMY172 grown in 2% lactate to an OD_{600} of 0.6. Proteins were electrophoresed on an SDS gel, transferred to a nylon membrane and probed with antisera against F_1 β-subunit. The result is shown in Plate 5.3. It is difficult to tell whether the amount of β-subunit in the suppressor strain (Lane 1) is increased over wild type (Lane 4). However, any possible increase in the total amount produced is very small compared to the amount expressed from the multicopy vector which does give pseudosuppression (Plate 4.4). An increase in the amount of β-subunit reaching the matrix is anticipated to account for the increase ATPase activity, but this may be difficult to detect using Western blots.

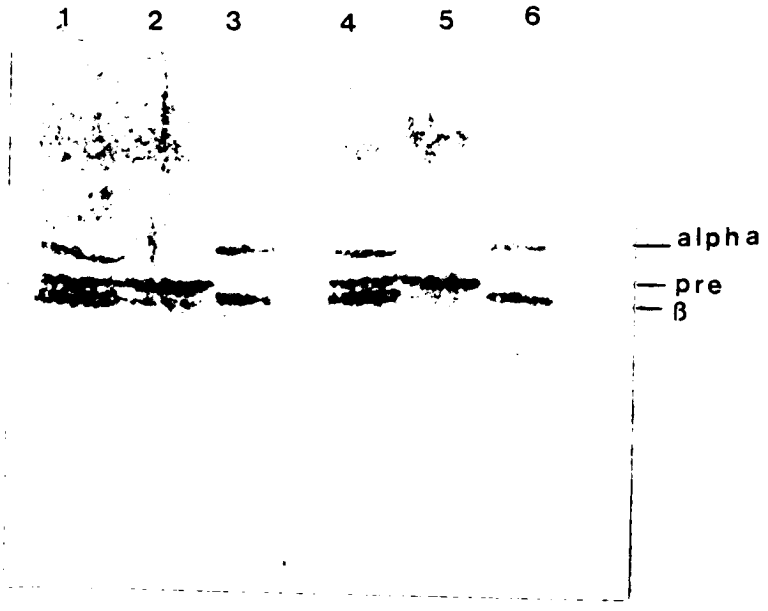
5.5 Biochemical Characterization of *mts1*

5.5.1 Effect of a Temperature Upshift

The temperature sensitive nature of *mts1* causes the strain carrying the mutation to fail to grow at 35°C. This is possibly due to the inability of essential mitochondrial precursors to be imported as a result of the *mts1* gene product being non-functional at this temperature. It may therefore be possible to detect the accumulation of precursors in the yeast cytoplasm after shifting the temperature to 35°C.

After growing the strain carrying the *mts1* mutation (EMY272) and strain EMY21 at 25°C, the culture was split and the incubation temperature of half was rapidly increased to 35°C. Samples were taken after growth at 25°C and 35°C for 7 hours when an effect on growth rate at 35°C of EMY172 compared to EMY21 was observed. Proteins were extracted as described in

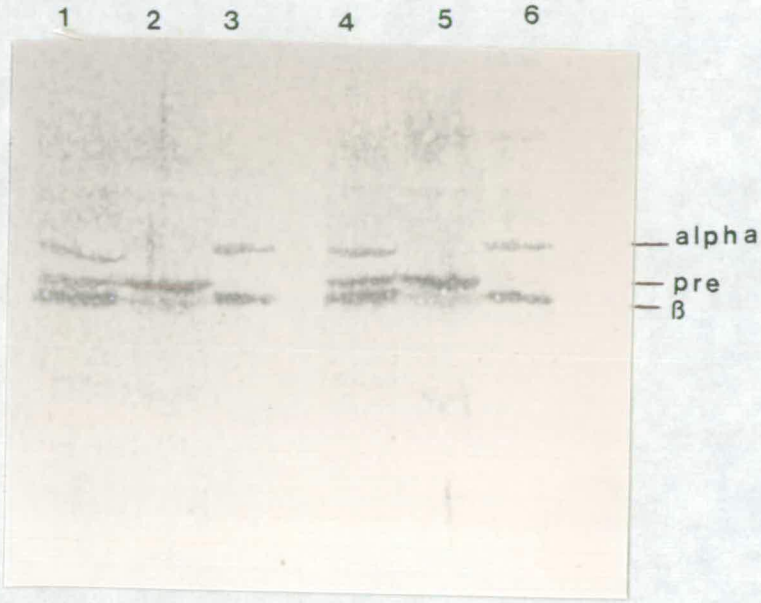
Plate 5.3 Comparison of β-subunit Precursor in Mutant and Wild-Type Cells.



Cells were grown in 2% lactate media at 25°C to an OD_{600} of 0.6. Mitochondria were prepared as described in Section 2.17, and the postmitochondrial supernatant was kept. Approximately 20 µg of Sphaeroplast protein and postmitochondrial supernatant protein, and 2 µg of mitochondrial protein from each strain was separated by SDS-PAGE and analyzed by Western Blots.

Lane	Strain	Relevant Genotype	Fraction
1	EMY21	*MTS1*, Δ2-15*atp2*	Sphaeroplast
2	EMY21	*MTS1*, Δ2-15*atp2*	Supernatant
3	EMY21	*MTS1*, Δ2-15*atp2*	Mitochondria
4	EMY172	*mts1*, Δ2-15*atp2*	Sphaeroplast
5	EMY172	*mts1*, Δ2-15*atp2*	Supernatant
6	EMY172	*mts1*, Δ2-15*atp2*	Mitochondria

Plate 5.3 Comparison of β-subunit Precursor in Mutant and Wild-Type Cells.



Cells were grown in 2% lactate media at 25°C to an OD_{600} of 0.6. Mitochondria were prepared as described in Section 2.17, and the postmitochondrial supernatant was kept. Approximately 20 µg of Sphaeroplast protein and postmitochondrial supernatant protein, and 2 µg of mitochondrial protein from each strain was separated by SDS-PAGE and analyzed by Western Blots.

Lane	Strain	Relevant Genotype	Fraction
1	EMY21	*MTS1*, Δ2-15*atp2*	Sphaeroplast
2	EMY21	*MTS1*, Δ2-15*atp2*	Supernatant
3	EMY21	*MTS1*, Δ2-15*atp2*	Mitochondria
4	EMY172	*mts1*, Δ2-15*atp2*	Sphaeroplast
5	EMY172	*mts1*, Δ2-15*atp2*	Supernatant
6	EMY172	*mts1*, Δ2-15*atp2*	Mitochondria

Section 2.11, and were separated by SDS-PAGE. The Western blots in Plate 5.4 show no increase in the cytoplasmic accumulation of mutant β-subunit precursor at the non-permissive temperature in the mutant strain. However, in both the wild-type and mutant strains, the amount of mutant precursor decreases after growth at 35°C without an increase in the amount of mature protein, suggesting that one effect of the heat-shock may be an increase in the rate of cytosolic proteolysis. The mutant strain appears to show a greater reduction in the amount of mature protein reaching the mitochondria, indicating perhaps a greater increase in proteolysis of the precursor in this strain compared to wild-type. This observation is compatible with the idea that the deleted precursor is remaining in the cytoplasm at the non-permissive temperature due to a block in import in the mutant strain, and is therefore more susceptible to proteolysis.

A decrease in the amount of cytochrome b_2 reaching the mitochondria in the mutant strain at the non-permissive temperature was not detected using Western blots (data not shown). It was not possible to examine the fate of other mitochondrial precursors but it is possible that the import of essential precursors may be affected upon temperature upshift and this would account for the temperature-sensitive phenotype.

5.5.2 Cell Location of the *MTS1* Gene Product.

At this stage it was not known whether the product encoded by the *MTS1* gene lay in a mitochondrially-located component or in a cytoplasmic component. If mitochondria from the *mts1* strain were able to suppress the import of the Δ2-15 precursor *in vitro*, then this would perhaps indicate that the mutation lay in a mitochondrially-located component. In order to test this, mitochondria were isolated from wild-type and from *mts1* yeast, and were used in an *in vitro* import assay as described in Section 2.17, using wild-type or Δ2-15 precursors. The results in Plate 5.5 do not show any improvement in processing of the deletion precursor (Lane 18), nor is any protected from externally added protease (Lane 20). There is however an apparent increase

Plate 5.4 Effect of Increase in Temperature on β-subunit Precursor in Mutant and Wild-type Cells.

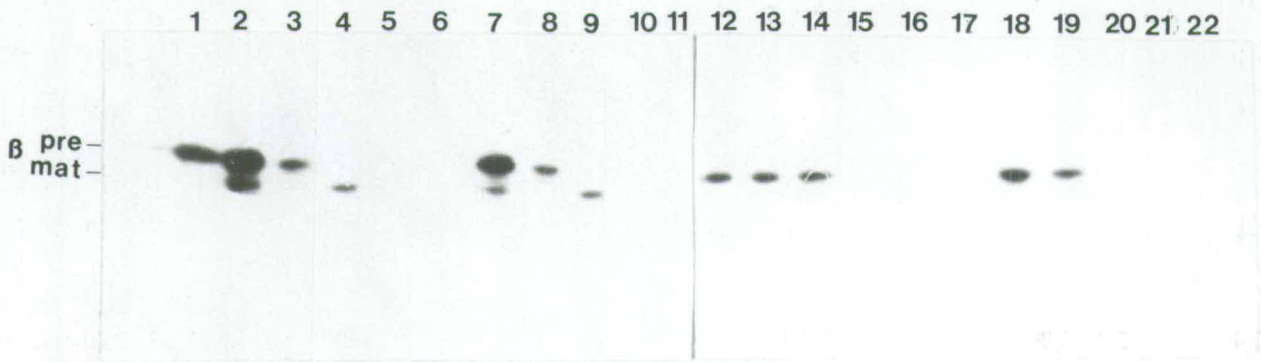
Mutant and wild-type strains were grown in SD to an OD_{600} of 0.5, the cultures were split and diluted with an equal volume of SD at either 25°C or 45°C to give final temperatures of 25°C and 35°C respectively. The cultures were grown shaking to an OD_{600} of approximately 0.7, at which point the mutant strain was growing more slowly than the wild-type at 35°C.

Lane	Strain	Relevant Genotype	Temperature
1	EMY21	*MTS1*, Δ2-15*atp2*	25°C
2	EMY21	*MTS1*, Δ2-15*atp2*	35°C
3	EMY172	*mts1*, Δ2-15*atp2*	25°C
4	EMY172	*mts1*, Δ2-15*atp2*	35°C

Plate 5.5 *In vitro* **Suppression.**

Mitochondria were isolated from mutant (EMY172) and wild-type (EMY21) strains, as described in Section 2.17, and were incubeted with mutant (Δ2-15) and wild-type ($F_1\beta$) in vitro synthesized radiolabelled precursor proteins for 30 minutes as described in Section 2.18. After incubation with Proteinase K (10 μg/ml) or Proteinase K and Triton (0.3%), mitochondria were reisolated, proteins were separated by SDS-PAGE and detected by fluorography.

	LANE			
Precursor	**β-subunit**		Δ2-15	
Mitochondrial Source Strain	**EMY21**	**EMY172**	**EMY21**	**EMY172**
Translation	1		12	
Mitochondria	2	7	13	18
Supernatant	3	8	14	19
Mitochondria + Proteinase K	4	9	15	20
Supernatant + Proteinase K	5	10	16	21
Mitochondria + Proteinase K + Triton	6	11	17	22


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
β pre
mat

in the association of the mutant precursor to the mitochondria (Lane 18). This improvement is not observed with the wild-type precursor. The increase interaction may mean that the *MTS1* product is a mitochondrially-located receptor. However, such a small increase could be accounted for by the carry-over of a loosely-associated cytoplasmic protein in the mitochondria isolation procedure.

5.6 DISCUSSION.

A yeast strain carrying the *mts1* temperature sensitive mutation has been isolated which is able to suppress a defective mitochondrial targeting sequence. Because of the way in which it was isolated, the *mts1* mutation is proposed to lie in a component of the protein targeting apparatus. The suppressor mutation is in a nuclear gene, and is genetically-linked to the temperature-sensitivity. The temperature-sensitive phenotype is more pronounced on glucose plates than on glycerol plates. It is possible that the expression of the putative import component may be reduced on fermentable carbon sources such as glucose, as only a minimal amount would be needed to import the reduced amount of mitochondrial precursors produced when the cell was not respiring. Under these circumstances, the lethal effects of the component's instability at higher temperatures may be more pronounced than if there was an abundance of the component in the cell.

As the original screen was carried out by looking for improved growth on glycerol plates, the mutated component must still be able to interact with the wild type presequences of other proteins as well as accomodating the defective targeting sequence. This is not too surprising, considering that within the cell, targeting components must be able to interact efficiently with a wide variety of wild-type precursors with a diverse range of presequences. Also, the mutant targeting sequence is only partially defective, and therefore would only require a very subtle increase in the efficiency of interaction to allow supression. The β-subunit has been demonstrated to contain functionally-redundant targeting information (Bedwell *et al.,* 1987), and it is

possible that the increased interaction of the *MTS1* gene product with the 2-15 deletion approaches the efficiency of interaction seen with the 16-35 deletion precursor discussed in Chapter 4.

The nature of the suppression caused by *mts1* has been partly defined. It is clear that the *mts1* mutation does not act by increasing the levels of mutant precursor within the cell. However, steady state levels of mature β-subunit in the mitochondria do not appear to increase to any great extent, suggesting that it is the efficiency of targeting which may be affected, rather than overall levels. The effect of an increase in temperature on the mutant strain suggests that the targeting of the deletion β-subunit is being affected as the amount of mature β-subunit reaching the mitochondria is reduced. Pulse-labelling of yeast cells will allow the fate of precursors over time to be examined and will hopefully clarify this observation. It is probable that the mutation is not acting at the mitochondrial surface, as mitochondria from the mutant do not import the defective precursor any more efficiently than wild-type mitochondria. However, an increase in binding is observed, and the *in vitro* assay could be used to identify the component causing increased binding from yeast lysate.

As the full nature of the *mts1* mutation is not apparent, the cloning of the wild-type *MTS1* gene should enable its role in targeting to be identified.

CHAPTER SIX

CLONING AND SEQUENCING OF THE *MTS1* GENE

6.1 INTRODUCTION

The isolation of a mutation in *Saccharomyces cerevisiae* which suppresses the defective Δ2-15 deleted presequence of the β-subunit as described in the Chapter 5, was the initial step in the identification of components of the targeting pathway. Several yeast genes whose products are involved in mitochondrial protein targeting have previously been identified. The *mas1* and *mas2* mutations which result in the accumulation of the precursor form of a mitochondrial protein (Yaffe and Schatz, 1984) have allowed the wild-type genes to be cloned from *S. cerevisiae* (Jensen and Yaffe, 1988). These genes are known to encode the processing protease. Other yeast genes involved in the targeting pathway include *SSA1, SSA2, SSA3* and *SSA4,* and their cloning and sequencing has confirmed that they encode hsp70 heat shock proteins (Werner-Washburne *et al.*, 1987). The *mif4* mutation affects assembly of proteins in the mitochondrial matrix and encodes an Hsp60 protein.

Cloning of the wild type *MTS1* gene should help to define its possible role in targeting. Subsequent sequence analysis of the *MTS1* gene, and database searches should reveal whether the gene is already known, and is either involved in mitochondrial protein targeting, or to have some other function within the cell; or whether it is a novel sequence which encodes a product whose function can be inferred by its relatedness to other known genes.

As the *mts1* mutation is genetically linked to the temperature sensitive phenotype (Section 5.3.2), and is therefore presumably within the same locus, the cloning of the wild type gene by complementation of the temperature sensitive phenotype was attempted.

6.2 Screening of a Yeast Genomic Library for Clones which Complement the *mts1* Temperature Sensitive Mutation.

The library in YEp13 was originally constructed by Broach *et al.,* (1979) by partial digestion of total yeast genomic DNA with *Sau3A* and ligation into the *BamHI* site of YEp13. DNA was prepared from *E. coli* cells containing the

plasmid library by plating out 10^6 cells onto 10 LB+amp plates, pooling the cells and then growing them through 2 generations in 1 litre of LB+amp. DNA was prepared by the alkaline lysis method as described in Section 2.6.1.2. The strain EMY53 (*mts1, ura3, leu2*; Table 2.1) was transformed using 5 μg library DNA to *LEU*$^+$ using the lithium acetate procedure as described in Section 2.5.2, except the heat shock step was omitted, as this adversely affects the viability of the temperature sensitive cells (data not shown). Plates were incubated at 25°C for two days to allow cells to recover from the transformation procedure, and were then shifted to 35°C for a further three days to select for clones which could complement the temperature sensitive defect. Colonies which had grown at the non-permissive temperature were picked onto fresh plates, and incubated at 35°C. This resulted in 32 putative positive clones, arising at a frequency of approximately 1 in 10^4 clones screened.

Plasmid DNA was isolated from 12 of the yeast that could grow at 35°C, as described in Section 2.6.3.1, and was used to transform *E.coli* strain MM294 (Section 2.5.1). Only plasmid DNA from 3 of the yeast colonies was able to transform the *E.coli* to ampicillin resistance. Plasmid DNA was extracted from the 3 *E. coli* transformants (Section 2.6.2.1) The 3 plasmids were then subjected to restriction endonuclease digestion, and the pattern analyzed by agarose gel electrophoresis. The results of this initial screen for inserts of yeast chromosomal DNA showed that two of the plasmids have inserts which are similar though not identical and the third plasmid has a different insert. Further analysis of this third plasmid revealed no similarity to the parental YEp13 vector and it was not studied further. The two similar plasmids were named pEE23 and pEE24.

To verify that the plasmids could complement the temperature sensitive phenotype, EMY53 (*mts1*) was transformed with plasmids pEE23 and pEE24. The results shown in Table 6.1 confirm that it is the plasmids which allow the strain to grow at 35°C and that the complementation phenotype of the original transformants was not due to a reversion of the *mts1* mutation.

Table 6.1 Plasmids pEE23 and pEE24 Can Complement the Temperature Sensitive Phenotype in *mts1*.

Strain	Relevant Genotype	Plasmid	25°C	35°C
EMY53	*mts1*	-	+ + +	-
EMY53	*mts1*	pEE23	+ + +	+ + +
EMY53	*mts1*	pEE24	+ + +	+ + +
EMY53	*mts1*	YEp13	+ + +	-

The strain EMY53 is an outcross of the strain carrying the mts1 mutation (EMY172). Plasmids pEE23 and pEE24 isolated from the original screen for complementation were transformed into EMY53, and the phenotypes of the resulting strains were analyzed by spotting on YPD plates and incubating for two days.

6.3 Characterization of Complementing Clones.

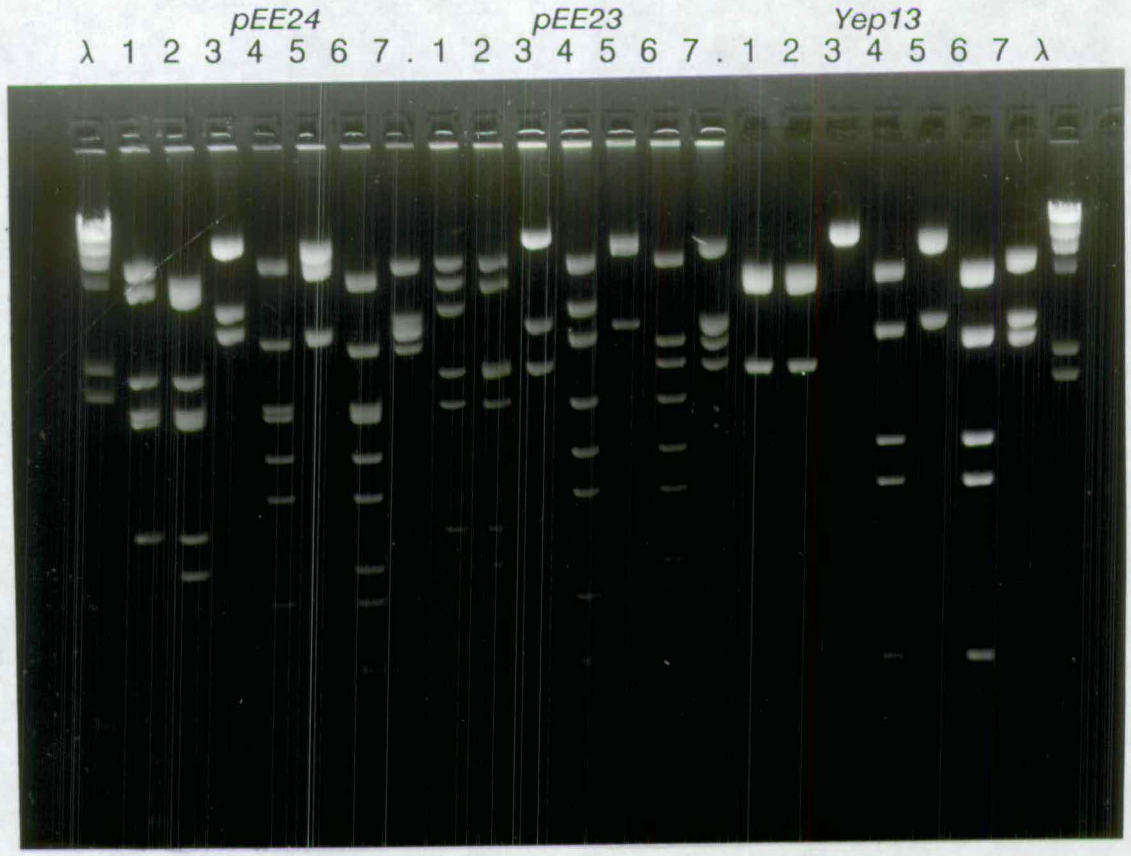
Further restriction endonuclease digestion analysis of the two plasmids pEE23 and pEE24 was carried out to establish the similarity of the complementing clones to each other. The results in Plate 6.1 confirm that each fragment contains similar restriction sites, suggesting that pEE23 and pEE24 contain the same fragment of chromosomal DNA. Digestion with *BamHI* revealed that plasmid pEE23 has *BamHI* sites regenerated at both ends of the insert. This was not the case for the plasmid pEE24, where neither of the *BamHI* sites was regenerated. Also, the fragment from pEE23 contains a *PstI* site which is not present in the fragment in pEE24. These differences suggest that although the inserts contain essentially the same region of chromosomal DNA, the two isolates are independent and were generated by digestion at different *Sau3A* sites.

A restriction map of the 7.5 kb *BamHI* fragment of complementing DNA originating from the plasmid pEE23 was determined by single and double digestions. This is shown in Figure 6.1.

6.3.1 Definition of the Minimum Complementing Region.

To determine the minimum complementing region, fragments generated by restriction endonuclease digestion of the 7.5 kb *BamHI* fragment were subcloned into the pFL38 vector (Figure 2.10) as depicted in Figure 6.1. These plasmids were transformed into strain EMY53 and complementation of the temperature sensitive defect was analyzed. None of the smaller fragments was able to complement the temperature sensitive phenotype, suggesting that at least one of each of the sites for the restriction enzymes used lies in the DNA coding for the gene or in a regulatory region. As neither of the two larger *BglII* fragments complemented, it was probable that the *BglII* sites lay in the gene. To verify this, the 7.5 *BamHI* fragment was subcloned into the vector pTZ18R to give plasmid pEE42, cut with *BglII*, and religated, thus removing the small *BglII* fragment between the *BglII* sites. The 7.5 kb *BamHI* fragment with the 120bp *BglII* fragment removed was then sub-cloned into

Plate 6.1 Restriction Endonculease Digestions of Complementing Clones.



KEY

1 - EcoRI

2 - EcoRI + HindIII

3 - HindIII

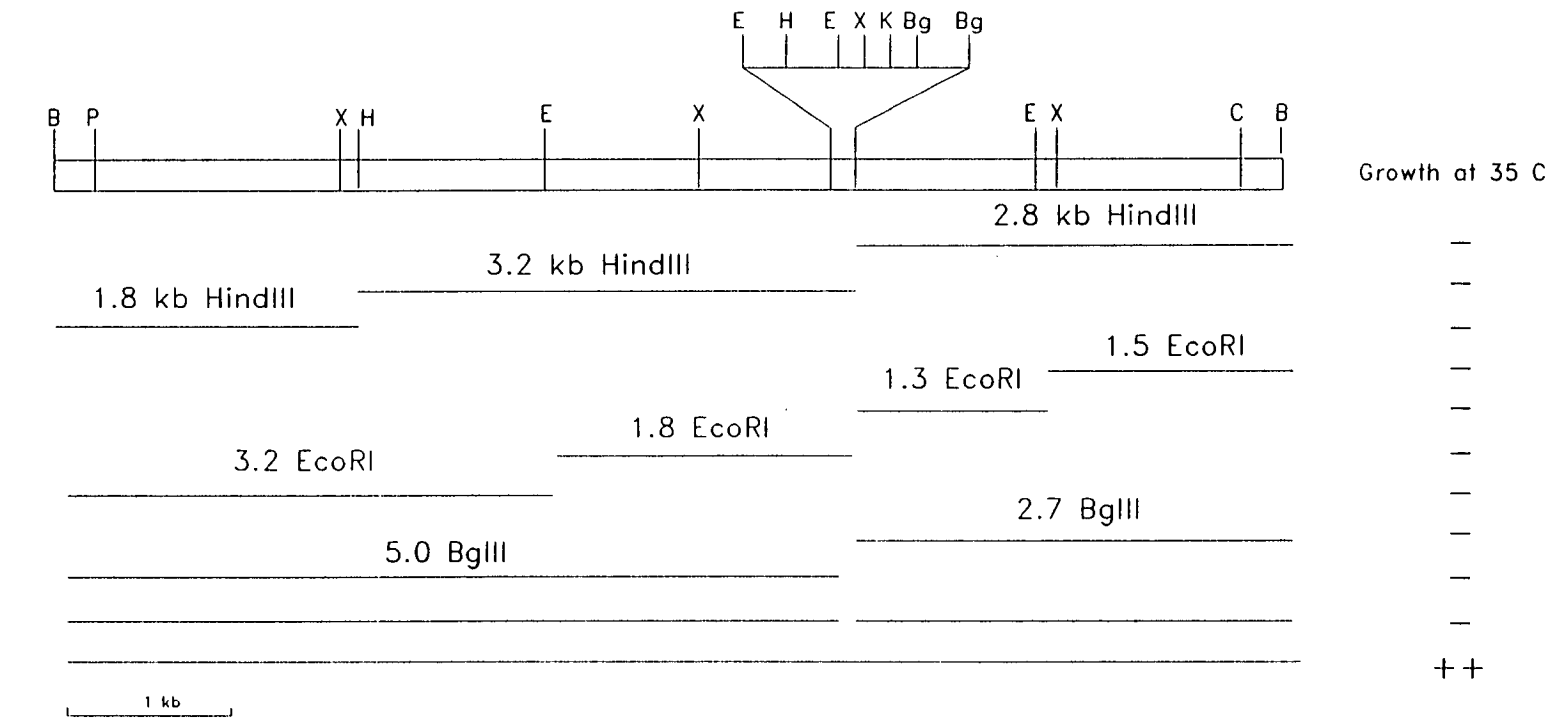
4 - EcoRI + BglII

5 - BglII

6 - EcoRI + BglII + HindIII

7 - HindIII

Figure 6.1 Restriction Map and Complementation Map of 7.5 kb BamHI Fragment which Complements mts1.



B - *BamHI* P - *PstI* X - *XbaI* H - *HindIII*
E - *EcoRI* K - *KpnI* Bg - *BglII* C - *ClaI*

pFL38, and used to transform EMY53. The inability of this plasmid to complement the temperature sensitive phenotype suggests that at least one of the *BglII* sites lies in the coding region or a regulatory region of the complementing gene.

6.3.2 Confirmation that the Complementing DNA Contains the *MTS1* Gene.

As the complementing DNA was originally cloned in a 2μ-based (multicopy) vector, it was possible that the complemention observed was due to a mass-action suppressor. Any gene product of the complementing DNA would be at elevated levels due to the high copy number of the plasmid. To test this possibility the 7.5 kb *BamHI* fragment was subcloned from pEE23 into pFL38 (*ARS-CEN* based) and pFL44 (2μ-based) (Figure 2.10) to give plasmids pEE26 and pEE27 respectively. The transformation of EMY53 with these two plasmids revealed that the 7.5 kb *BamHI* fragment of DNA could complement the temperature sensitive defect at low copy number (pEE26) as well as at high copy number (pEE27). This suggests that only a single copy of the gene is required for complementation, and that it is unlikely to be a mass-action suppressor.

However it was considered possible that the complementing DNA was acting by suppressing the *mts1* defect, rather than true allelic complementation. For example a regulatory protein which increased the level of the *mts1* gene product in the cell might allow sufficient to be functional for growth even at the non-permissive temperature. Alternatively, it could encode an accessory protein which stabilizes the *mts1* gene product at the non-permissive temperature. It would be unlikely for a gene which complemented in this way to be genetically linked to the original *mts1* mutation. Integration of the complementing DNA at its chromosomal locus and subsequent crosses would determine whether or not this was the same locus as the *mts1* gene.

The complementing 7.5kb *BamHI* fragment was subcloned into the vector YIp5 (Figure 2.11), which contains no *ARS-CEN* or 2μ sequences. The

plasmid (pEE46) was cut with the restriction enzyme *BglII* which cuts internally to the fragment. This would yield ends which could recombine with the chromosomal copy of the cloned gene. The linearized DNA was introduced into a wild-type (DBY747) and *mts1* (EMY53) mutant background, and integrants were selected using the co-integrated *URA3* marker to give strains EMY63 and EMY64 respectively.

To confirm the integration of the complementing DNA, chromosomal DNA was prepared from the integrant and wild-type strains and digested with the restriction endonuclease *PstI*. After separation on an agarose gel, the DNA was transferred to a nylon mebrane by Southern blotting (Section 2.10.1.1), and probed with the 1.8 kb *EcoRI* fragment from pEE23. The result shown on Plate 6.2 confirms that the complementing DNA occurrs at a single locus, and that the strains EMY63 and EMY64 are integrants at this locus.

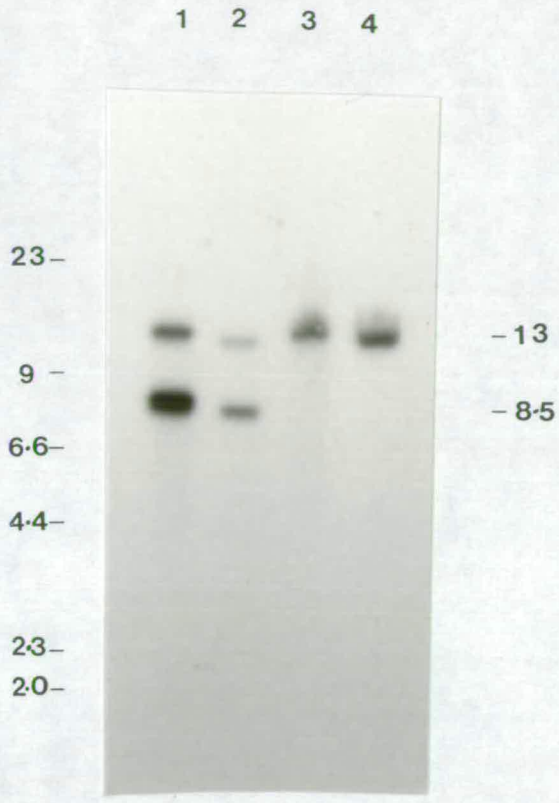
The two strains EMY63 and EMY64 were crossed with the appropriate strain of the opposite mating type to give heterozygous diploids of the *MTS1* locus. The four parental and two diploid strains were compared for their ability to grow at the non-permissive temperature. The results in Table 6.2 demonstrate that the integration event has resulted in complementation of the *mts1* temperature sensitive phenotype.

The two diploid strains (EMY65, EMY66) were induced to sporulate (Section 2.18.3), and their progeny were examined after tetrad dissection. The results (Table 6.3) show that the all temperature sensitive progeny of the strain EMY65 are also URA$^-$, demonstrating that the integrated complementing DNA and *URA3* gene always segregate with the wild-type *MTS1* allele, and never with the *mts1* allele. Obversely, none of the progeny of EMY66 are temperature sensitive, demonstrating that in this case the *mts1* allele always segregates with the integrated complementing DNA. These results indicate that the cloned DNA has integrated at the *mts1* or *MTS1* locus and means that it is likely that the cloned DNA does contain the wild-type copy of the *MTS1* gene, and not an unlinked suppressor.

Plate 6.2 Southern Blot of Integrants of Complementing Fragment.

Chromosomal DNA was prepared from yeast strains grown in YPD to an OD_{600} of 0.7. Approximately 2 µg DNA was digested with the restriction enzyme PstI which cuts once in the complementing DNA (see Diagram). The DNA was electrophoresed on a 0.5% agarose gel and transferred to a nylon membrane. The blot was probed with the 1.8kb EcoRI fragment from pEE42 (Figure 6.2), labelled with ^{32}P, and washed at high stringency (0.1 x SSC) before autoradiography.

Lane	Strain
1	*EMY64 (mts1::pEE46)*
2	*EMY63 (MTS1::pEE46)*
3	*EMY53 (mts1)*
4	*DBY747 (MTS1)*


1 2 3 4
23–
9 –
6·6–
4·4–
2·3–
2·0–
–13
–8·5

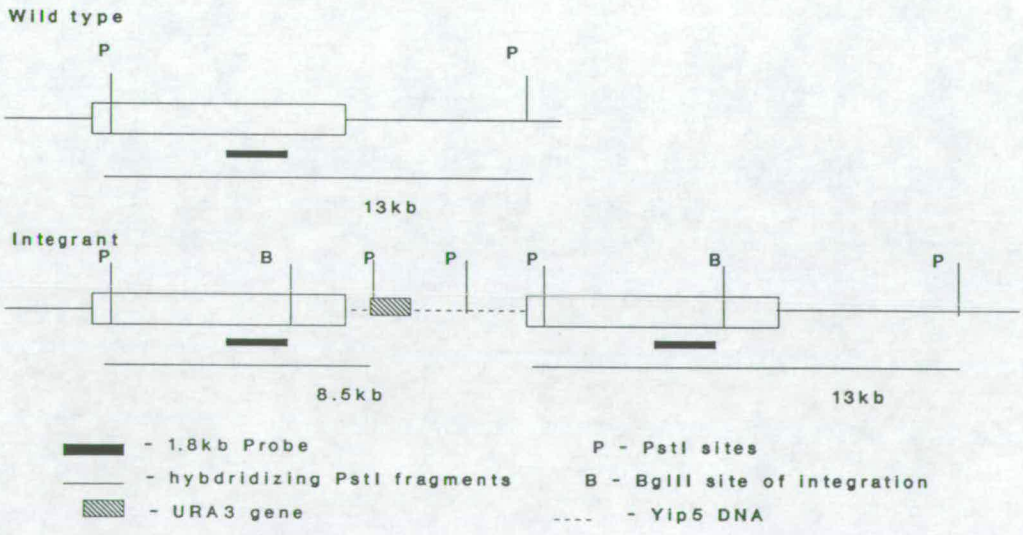

Wild type
P
P
13kb
Integrant
P
B
P
P
P
B
P
8.5kb
13kb
- 1.8kb Probe
- hybdridizing PstI fragments
- URA3 gene
P - PstI sites
B - BglII site of integration
- Yip5 DNA

Table 6.2 Phenotype of Integrants of Cloned DNA.

Strain	MAT	Genotype	25°C	35°C	URA
EMY53	a	*mts1, ura3*	+ + +	-	-
DBY747	α	*MTS1, ura3*	+ + +	+ + +	-
EMY63	α	*MTS1::pEE46, ura3*	+ + +	+ + +	+ + +
EMY64	a	*mts1::pEE46, ura3*	+ + +	+ + +	+ + +
EMY65	a/α	*ura3, MTS1::pEE46* *ura3, mts1*	+ + +	+ + +	+ + +
EMY66	α/a	*ura3, mts1::pEE46,* *ura3, MTS1*	+ + +	+ + +	+ + +

Integrants at the MTS1 locus were constructed by transforming wild-type and mts1 mutants with a linear fragment containing the cloned MTS1 gene and the URA3 gene in a Ylp5 based plasmid, pEE46. Heterozygous diploids were constructed by mating EMY53 and EMY63 (EMY65), and DBY747 and EMY64 (EMY66). Strains were spotted on the appropriate plate and incubated for 3 days.

Table 6.3 The Cloned DNA Integrates at the *MTS1* Locus

a. EMY65 a/α *his3-D1 HIS4 leu2-3 leu2-112 ura3 trp1 MTS1::pEE46*
HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 mts1
pEE46 - *MTS1,URA3*

Tetrad	Spore	25°C	35°C	URA+	Tetrad	Spore	25°C	35°C	URA+
1	a	+	+	+	6	a	+	+	+
	b	+	-	-		b	+	+	+
	c	-	-	-		c	+	-	-
	d	-	-	-		d	+	-	-
2	a	+	+	+	7	a	+	+	+
	b	+	-	-		b	+	+	+
	c	+	-	-		c	+	-	-
	d	-	-	-		d	-	-	-
3	a	+	+	+	8	a	+	-	-
	b	+	+	+		b	-	-	-
	c	+	-	-		c	+	+	+
	d	-	-	-		d	+	+	+
4	a	+	+	+	9	a	+	-	-
	b	+	-	-		b	+	+	+
	c	+	+	+		c	-	-	-
	d	-	-	-		d	-	-	-
5	a	+	-	-	10	a	+	+	+
	b	+	+	+		b	-	-	-
	c	-	-	-		c	-	-	-
	d	-	-	-		d	-	-	-

b. EMY66 a/α *his3-D1 HIS4 leu2-3 leu2-112 ura3-52 trp1 mts1::pEE46*
HIS3 his4 leu2-3 leu2-112 ura3-52 TRP1 MTS1
pEE46 - *MTS1,URA3*

Tetrad	Spore	25°C	35°C	URA+	Tetrad	Spore	25°C	35°C	URA+
1	a	+	+	+	6	a	+	+	+
	b	+	+	+		b	+	+	-
	c	+	+	-		c	-	-	-
	d	+	+	-		d	-	-	-
2	a	+	+	+	7	a	+	+	+
	b	+	+	-		b	-	-	-
	c	+	+	-		c	+	+	-
	d	-	-	-		d	-	-	-
3	a	+	+	+	8	a	+	+	-
	b	+	+	+		b	+	+	-
	c	+	+	-		c	-	-	-
	d	-	-	-		d	+	+	+
4	a	+	+	-	9	a	+	+	+
	b	+	+	+		b	+	+	+
	c	+	+	+		c	+	+	-
	d	+	+	-		d	+	+	-
5	a	+	+	-	10	a	+	+	+
	b	+	+	+		b	+	+	-
	c	+	+	-		c	+	+	+
	d	+	+	+		d	-	-	-

Progeny were tested for their phenotypes by spotting on appropriate plates and incubating for two days at the appropriate temperature.

6.4. Characterization of the Cloned *MTS1* Gene.

6.4.1 The *MTS1* Gene is Essential.

The temperature-sensitive nature of the *mts1* mutation suggests that the product of the *MTS1* gene is essential for growth. To demonstrate this, the cloned *MTS1* gene was disrupted with the *URA3* gene by inserting a *BglII* fragment containing the *URA3* gene from pFL38 into the *BglII* site of pEE42. The resulting plasmid, pEE47, was digested with *PstI* and *ClaI* which cut at unique sites within the cloned fragment (Figure 6.1; Plate 6.3). The cut plasmid was transformed into a heterozygous (*MTS1/mts1*) diploid (EMY67) constructed by mating DBY747 (*MTS1*) with EMY53 (*mts1*), and the resulting transformants were examined for their ability to grow at the non-permissive temperature. To confirm that the *mts1* gene has been disrupted in the heterozygous diploid, one transformant was picked which was not temperature sensitive (EMY68), and chromosomal DNA was extracted. The DNA was cut with either EcoRI or HindIII, and a Southern blot probed with the 1.3 kb EcoRI fragment of the complementing DNA (Figure 6.1) is shown in Plate 6.3. This shows that one allele of the *MTS1* locus has been disrupted.

Sporulation of the diploid EMY68 followed by random spore anaylsis (Table 6.4) revealed that no haploid progeny were found which were URA$^+$, suggesting that the disruption of the *mts1* gene by the *URA3* gene has rendered the progeny non-viable. This confirms that the *MTS1* gene has an essential function within the cell. None of the viable progeny were temperature sensitive, thus confirming that the cloned DNA has disrupted the mutant *mts1* allele, and therefore contains the *MTS1* gene.

6.4.2 Northern Analysis of *MTS1* Transcript.

To further characterize the *MTS1* gene and its product, RNA was prepared from the wild-type strain DBY747 grown in YPD (Section 2.6.4), electrophoresed on a denaturing agarose gel (Section 2.9.3) and transferred to nylon membrane (Section 2.10.1.2). The Northern blot was probed with the 1.8kb *EcoRI* fragment from the cloned DNA (Figure 6.1), and the result in

Plate 6.3 Southern Blot of Disruptants

Chromosomal DNA was prepared from yeast strains grown in YPD to an OD_{600} of 0.7. Approximately 2 µg DNA from each strain was digested with either EcoRI and HindIII. The DNA was electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane. The blot was probed with the 1.3 kb EcoRI fragment from pEE38 (Figure 6.2), labelled with ^{32}P, and washed at high stringency (0.1 x SSC) before autoradiography.

Lane	Strain	Relevant Genotype	Digest
1	EMY68	*MTS1/mts1::URA3* diploid	*HindIII*
2	EMY68	*MTS1/mts1::URA3* diploid	*EcoRI*
3	EMY67	*MTS1/mts1* diploid	*HindIII*
4	EMY67	*MTS1/mts1* diploid	*EcoRI*

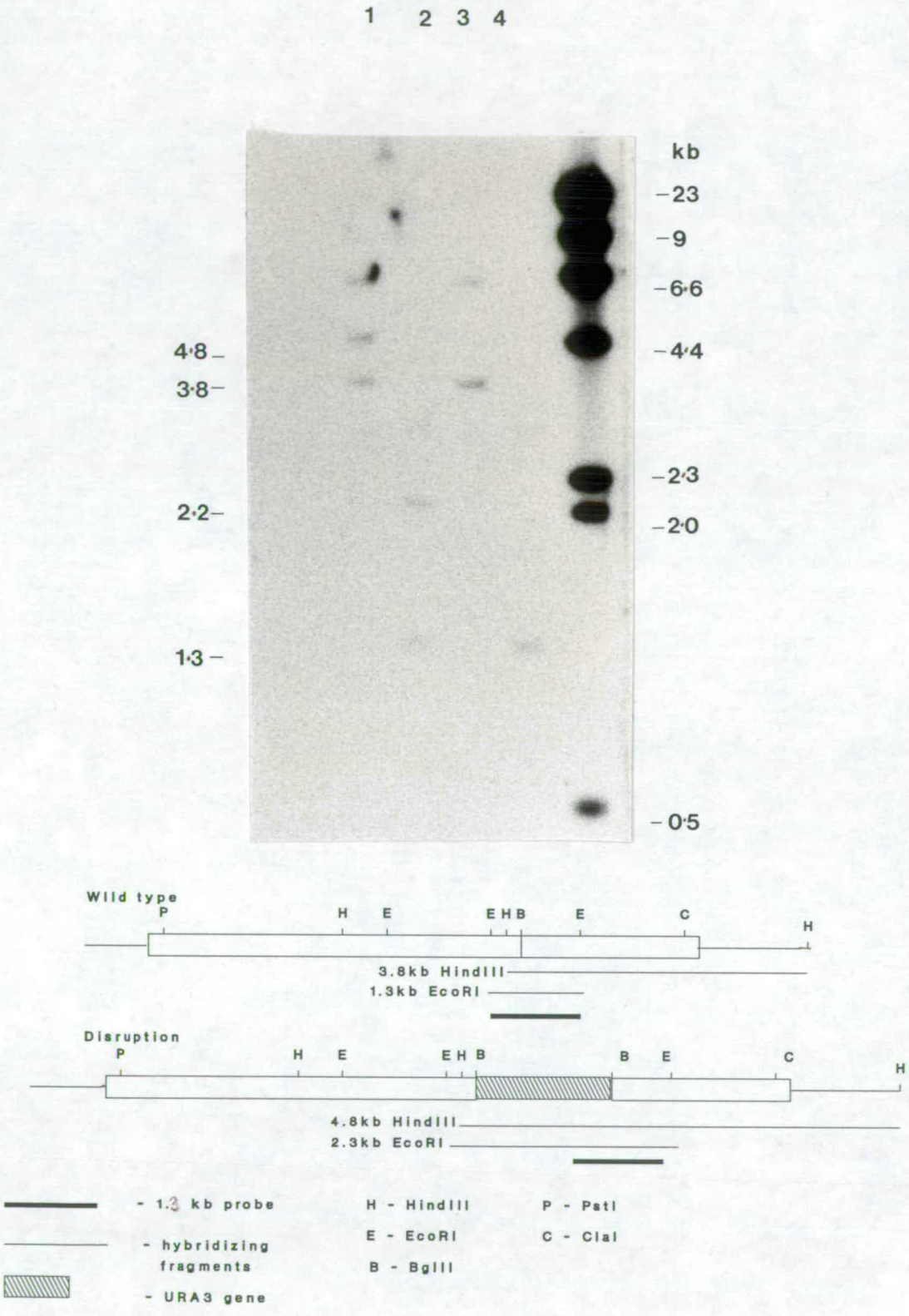

1 2 3 4
kb
23
9
6·6
4·4
2·3
2·0
0·5
4·8
3·8
2·2
1·3
Wild type
P
H
E
E H B
E
C
H
3.8kb HindIII
1.3kb EcoRI
Disruption
P
H
E
E H B
B
E
C
H
4.8kb HindIII
2.3kb EcoRI
- 1.3 kb probe
- hybridizing fragments
- URA3 gene
H - HindIII
E - EcoRI
B - BglII
P - PstI
C - ClaI

Table 6.4 The *MTS1* Gene is Essential

Strain	Relevant Genotype	Number of Progeny Tested	URA$^+$	ts
EMY68 (diploid)	*mts1::URA3* / *MTS1*	200	0	0

The diploid EMY67 was constructed by mating EMY53 with DBY747. Disruptants were obtained by transforming with linear DNA from plasmid pEE47 (MTS1::URA3) cut with PstI and ClaI. One non-temperature sensitive transformant (EMY68, URA$^+$) was grown in sporulating media, and random spore analysis was carried out. No haploid progeny were found which were URA$^+$.

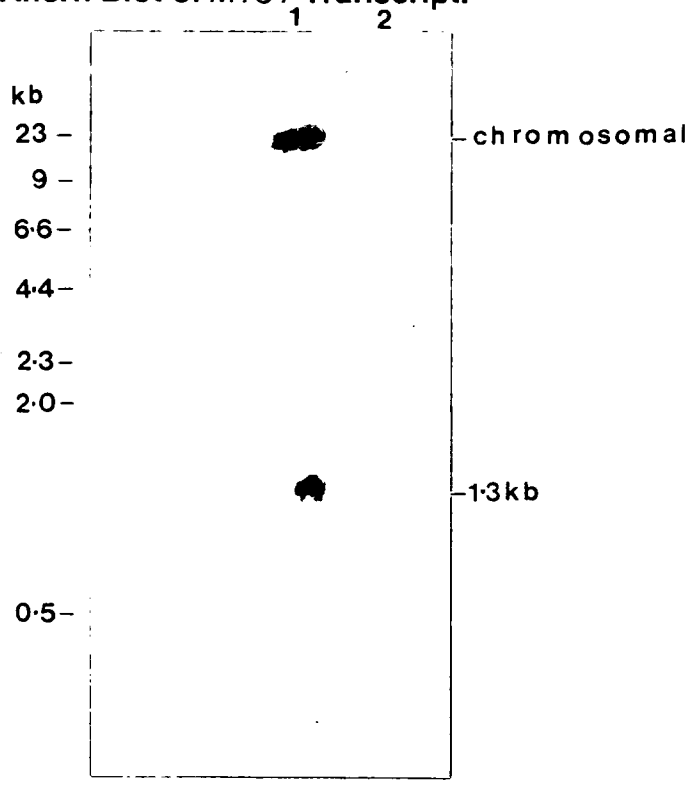
Plate 6.4 confirms the presence of a transcript of approximately 1.3 kb. As the *MTS1* gene is known to be located at least partially in the 1.8 kb fragment from complementation analysis, the 1.3 kb RNA band is presumably the transcript of the *MTS1* gene, although it is possible that the 1.8 kb fragment contains DNA from more than one gene.

6.5 Sequencing of the *MTS1* Gene

As it was known that a *BglII* site lay in the *MTS1* complementing DNA, and that this was adjacent to an *EcoRI* site (Figure 6.1), the two *EcoRI* fragments in proximity to the *BglII* site (1.3 kb and 1.8 kb) were subcloned into the vector pTZ18R (Figure 2.8) in both orientations to enable sequencing of the complementing region of DNA. The resulting plasmids are shown in Figure 6.2. It was not possible to obtain the reverse orientation of the 1.3 kb fragment using the insertional inactivation of the *lacZ* as a screen for the recombinant plasmid. This may be because β-galactosidase is being expressed from a fortuitous promoter and contiguous open reading frame in the cloned DNA. The entire 7.5 kb fragment was also cloned into pTZ18R in both orientations and these plasmids are illustrated in Figure 6.2.

DNA sequencing was initially carried out on the plasmids containing the *EcoRI* fragments (Figure 6.2) using reverse primer (RP) which would anneal to a site in the pUC18 polylinker of the pTZ18R vector upstream of the *EcoRI* site. Sequencing was carried out on single-stranded DNA as described in Section 2.8.2, which enabled approximately 300 bases to be read from the reverse primer site. In order to extend the sequence, oligonucleotide primers were synthesized corresponding to the determined sequence, and were used with single-stranded DNA prepared from the plasmids pEE42 and pEE42r which contain the 7.5 kb *BamHI* fragment in each orientation. The oligonucleotides used and a map showing their position in relation to restriction sites are shown in Figure 6.3. The nucleotide sequence determined is shown in Figure 6.4.

Plate 6.4 Northern Blot of *MTS1* Transcript.

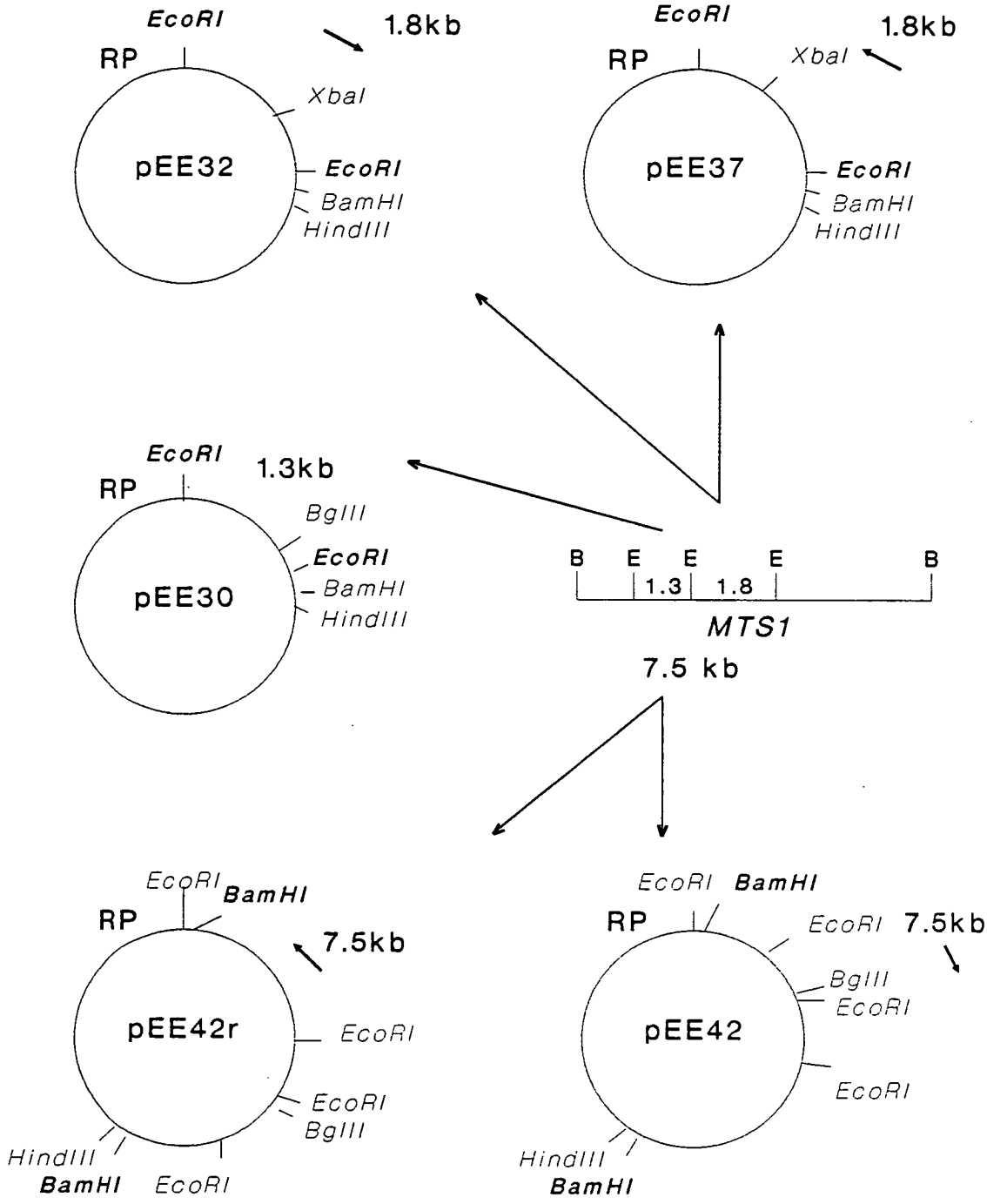


DNA or RNA was prepared from wild type yeast (DBY747) grown in YPD to and OD_{600} of 0.6. Approximately 5 µg RNA was electrophoresed on a denaturing gel, transferred to nylon membrane and probed with the 1.8kb EcoR1 fragment from pEE37 (Figure 6.2), labelled with ^{32}P. The blot was washed with a high stringency wash (0.1 x SSC) before autoradiograhpy.

1 - total nucleic acid

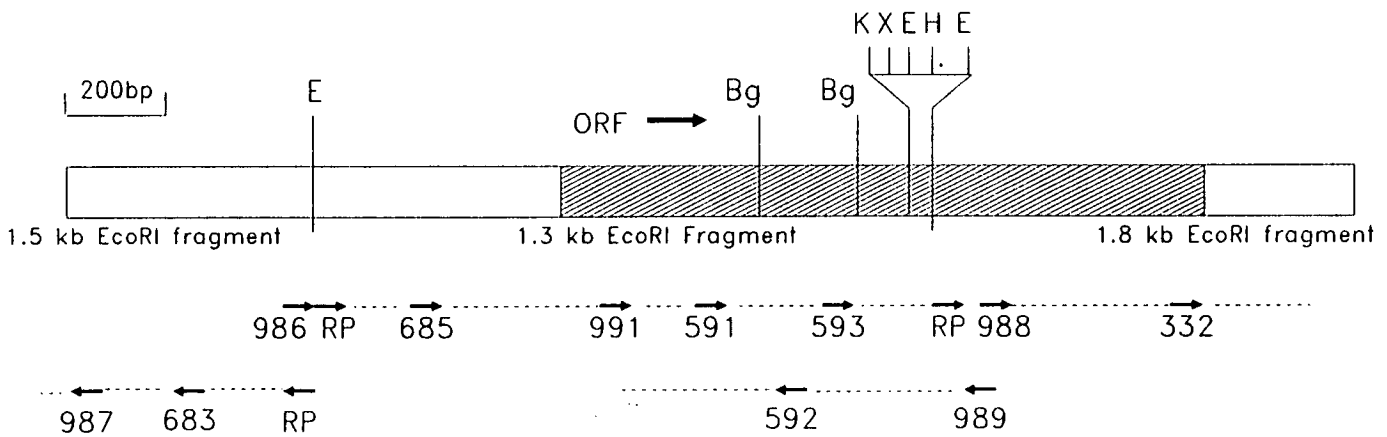
2 - chromosomal DNA

Figure 6.2 Plasmids Used in the Sequencing of the *MTS1* Gene



Plasmids were constructed by ligating fragments into the multiple cloning site of pTZ18r
E - EcoRI B - BamHI RP - Reverse primer site

Figure 6.3 Sequencing Map of MTS1 Gene



Oligonucleotide Primers:

RP	AACAGCTATGACCATG	987	CTTGAATCCGCTACTT
986	GAGTGCACCAAATCTAC	683	CTCATATTCTAGAGCA
685	ATATCTGTCAATGCTTG	592	CTTCAATGGCTTTGGCA
991	CAGTCGAAGAACAGCAC	989	GAGGATTGTCATCTCTT
591	TGTCCAAGACTACCAAG		
593	ACTTACCAGAAGGTTGT		
988	ATCAGAAGATCAAATAG		
332	CACGTGAAAGATCACAA		

Restriction Sites

E - *EcoRI*
Bg - *BglII*
K - *KpnI*
X - *XbaI*
H - *HindIII*

Figure 6.4 Nucleotide Sequence of *MTS1* Gene.

```
   1  TTATGCTCCA ACCTCCAACC CTCCTGATCA AAATCTTCTT GTTTAAGTAG
  51  CGGATTCAAG GTGGAGTCTA AATAAACACC TCTTAAATTA GCGAAATCTT
 101  GAGGGTTCGT AGTGGAAAAG GGAAAAAATG TATAATCTGG ACCTGTCATA
 151  GCGTTCATGA AATTAGCTAG AGATTTATTT AGCATTTTGA AAAAAGGGTC
 201  CCTAACTGGA TATTTAACAG ACCCACACAA CGTTGTATGC TCTAGAATAT
 251  GAGGGACCCC AGTGGAATCT GGAGGGTTGG TTTTAAAAGC AATGCTGAAC
 301  ACATTATTCT TGTCGTCTCT ATCAATATGC AAATGCTCGG CTCCTGTCTG
 351  GGAGTGCACC AAATCTACCG CAGTGAGTCT CAGCTCCGGA ACCGGTAGAA
 401  TTCTTCTCAC TTCATAACCA TGGAATATAC CTCCAACTGG ATATTTTGCT
 451  AACGGCCTGT GCTTGGGCAT ACGAGGACGC AAATCGCTGA AACCGCAACA
 501  TATATACCGC AGTATATTTT ATAGTAACTT TATTCTTTTG AGTAGCCTTC
 551  TAATTGGTGG CTTATTGATT ACAATTGCTT GTTATCATAT CTGTCAATGC
 601  TTGTTCAACA ATTCAATGAT TTACTACTAC CTTTATCATA GAGCCCCTCC
 651  GTATATTTAT CACTGTAAAC GAAGGGCAAA ATTTTTTACA TTCTTTTTTT
 701  TCGCCACCCA AAGGATTTAA AAAGGACATG AGAAAAATAA TTTCCTCTCT
 751  TCTAAATATA TATACTTTTG AAGGAATCAA AATTAAGCAA TTACGCTAAA
 801  ACCATAAGGA TAATGTCTGA AGCTCAAGAA ACTCACGTAG AGCAACTACC
 851  AGAATCTGTT GTCGATGCCC CAGTCGAAGA ACAGCACCAA GAACCACCAC
 901  AGGCTCCAGA TGCTCCACAA GAACCACAAG TTCCACAGGA ATCTGCTCCA
 951  CAGGAATCTG CTCCACAAGA ACCACCAGCT CCACAAGAAC AAAATGACGT
1001  TCCTCCACCA TCTAATGCTC CAATTTATGA AGGCGAAGAA TCCCACAGTG
1051  TCCAAGACTA CCAAGAGGCC CACCACGACC ACCAACCACC TGAACCCCAA
1101  CCATATTATC CTCCTCCTCC TCCAGGTGAA CACATGCACG GTCGCCCACC
1151  AATGCACCAC CGTCAAGAAG GAGAACTCTC GAACACCAGA TTGTTTGTTA
1201  GACCTTTCCC ATTGGACGTT CAAGAATCCG AGTTGAATGA AATCTTTGGT
1251  CCATTTGGAC CAATGAAGGA AGTCAAGATC TTGAACGGCT TCGCGTTTGT
1301  TGAATTTGAA GAAGCAGAAT CCGCTGCCAA AGCCATTGAA GAAGTTCACG
1351  GTAAGAGTTT TGCTAACCAA CCTTTGGAAG TTGTTTACTC TAAATTGCCT
1401  GCCAAGAGAT ACCGTATCAC CATGAAAAAC TTACCAGAAG GTTGTTCATG
1451  GCAAGATCTT AAAGATTTAG CCAGGGAAAA TAGTTTAGAA ACTACTTTTT
1501  CTAGCGTCAA TACCAGAGAT TTTGATGGTA CCGGTGCTCT AGAATTCCCT
1551  AGTGAAGAAA TCTTGGTCGA AGCTTTGGAG AGATTAAACA ATATTGAATT
1601  CAGAGGTTCT GTCATTACTG TTGAAAGAGA TGACAATCCT CCACCAATCA
1651  GAAGATCAAA TAGAGGTGGC TTCAGAGGTC GCGGCGGCTT CAGAGGCGGC
1701  TTCAGAGGTG GCTTCAGAGG CGGTTTCTCC AGAGGCGGCT TCGGTGGCCC
1751  CAGAGGTGGA TTTGGTGGTC CAAGAGGTGG TTACGGTGGC TATTCCAGAG
1801  GTGGCTACGG TGGCTACTCC AGAGGCGGAT ATGGTGGCTC CAGAGGTGGT
1851  TACGATAGTC CTAGAGGTGG TTACGATAGT CCAAGAGGTG GTTATTCCAG
1901  AGGTGGCTAT GGTGGTCCAA GAAATGATTA CGGTCCTCCA AGAGGTAGCT
1951  ACGGTGGTTC AAGAGGTGGT TATGATGGTC CAAGAGGCGA TTATGGTCCT
2001  CCAAGAGATG CATACAGAAC CAGAGATGCT CCACGTGAAA GATCACAACC
2051  AGGTAAGCCA TTTATATAGT TGAGAAAAAA AAAGGAGAAA TTAACAAAAG
2101  ATATGAATTG TTTTAAAACT TAAATATGAG ACTGAGAACC AATGAAAAAG
2151  GAAAACATCG TATTAAATGT TGAAAAAGTT TTTTTTTTGA AGAACTCCTC
2201  CCCATCCCTC CCCTCCCTTT TA
```

Key

EcoRI sites are in bold. The putative ATG (start codon) and TAG (stop codon) are in bold and underlined. Putative TAATA regions are underlined.

6.6 Analysis of *MTS1* Sequence

As discussed in Section 6.1, it was possible that the *MTS1* gene was a gene already known to be involved in mitochondrial protein targeting. However, comparison of the nucleotide sequence of *MTS1* to sequences in GenEmbl databases revealed no similarity to *MAS1, MAS2* or *hsp60*, and neither was it similar to any genes encoding proteins in other targeting pathways in yeast.

6.6.1. Identification of Open Reading Frame.

Within the *MTS1* sequence, an open reading frame encoding a protein of 418 amino acids was identified starting at the ATG at position 813 and terminating with the stop codon TAG at 2067. A putative TATA region was identified at 753. This would give rise to a transcript of approximately 1250 bases, which correlates with the transcript size observed on the Northern blot (Section 6.4.2; Plate 6.4). The deduced amino acid sequence from the open reading frame is shown in Figure 6.5 which would encode a protein of approximately 48 kDa, and is presumably the MTS1 protein.

6.6.2 Amino Acid Composition and Codon Usage.

The codon usage in the MTS1 protein was examined, and the codon bias was calculated (Table 6.5) and compared to that for highly expressed genes in yeast (Bennetzen and Hall, 1982). The codon bias of 0.539 suggests that the *MTS1* gene may be a relatively abundant protein, and compares well with that for iso-1-cytochrome c which is present in the cell as 0.5% total RNA (Bennetzen and Hall, 1982).

6.6.3 Structure of the *MTS1* Protein.

The MTS1 protein appears to have several distinct regions from inspection of the deduced amino acid sequence (Figure 6.5). The amino terminus contains many prolines with a high proportion of the acidic residues glutamate and aspartate. The carboxy terminus is very glycine rich, and the abundance of arginine residues means that it is more basic.

Figure 6.5 Deduced Amino Acid Sequence of MTS1 Open Reading Frame.

```
  1  MSEAQETHVE QLPESVVDAP VEEQHQEPPQ APDAPQEPQV PQESAPQESA
 51  PQEPPAPQEQ NDVPPPSNAP IYEGEESHSV QDYQEAHHDH QPPEPQPYYP
101  PPPPGEHMHG RPPMHHRQEG ELSNTRLFVR PFPLDVQESE LNEIFGPFGP
151  MKEVKILNGF AFVEFEEAES AAKAIEEVHG KSFANQPLEV VYSKLPAKRY
201  RITMKNLPEG CSWQDLKDLA RENSLETTFS SVNTRDFDGT GALEFPSEEI
251  LVEALERLNN IEFRGSVITV ERDDNPPPIR RSNRGGFRGR GGFRGGFRGG
301  FRGGFSRGGF GGPRGGFGGP RGGYGGYSRG GYGGYSRGGY GGSRGGYDSP
351  RGGYDSPRGG YSRGGYGGPR NDYGPPRGSY GGSRGGYDGP RGDYGPPRDA
401  YRTRDAPRER SQPGKPFI*
```

Table 6.5 Codon Usage in *MTS1* Gene.

Amino Acid	Preferred Codon(s) in yeast	No Used *MTS1*	Total in *MTS1*	Random Usage
Ala	GCU,GCC	18	21	10.5
Ser	UCU,UCC	16	29	9.67
Thr	ACU,ACC	9	9	4.5
Val	GUU,GUC	17	18	9
Ile	AUU,AUC	9	10	6.67
Phe	UUC	10	20	10
Tyr	UAC	11	20	10
Cys	UGU	1	1	0.5
Asn	AAC	5	14	7
His	CAC	11	11	5.5
Arg	AGA	31	37	6.17
Glu	GAA	38	42	21
Leu	UUG	8	16	8
Lys	AAG	5	9	4.5
Gly	GGU	40	63	15.75
Gln	CAA	17	21	10.5
Pro	CCA	36	52	13
Asp	No bias			
Met	Not degenerate			
Trp	Not degenerate			

TOTAL X = 282 Y = 393 Z = 151.76

$$\text{Codon Bias Index} = \frac{\text{(No Preferred Codons)-(Random Usage)}}{\text{(Total Codons)-(Random Usage)}}$$

$$= \frac{X-Z}{Y-Z} = \frac{282-151.76}{393-151.76}$$

$$= \frac{130.24}{241.24}$$

$$= \underline{\mathbf{0.539}}$$

The secondary structure of the proposed MTS1 protein was analyzed using protein secondary structure prediction programs available in the UWGCG package. PepPlot (Figure 6.6) is a plot of all the standard measurements of protein secondary structure. This includes the Chou and Fasman predictions for α-helices and β-sheets (Chou and Fasman, 1978), and the Garnier predictions for α and β structures (Garnier *et al.,*1978). Also shown is the hydropathy prediction of Kyte and Doolittle (Kyte and Doolittle, 1978). The PeptideStructure plots (Figure 6.7 and 6.8) shows regions of hydropathy superimposed on the Chou Fasman secondary structure predictions (Wolf *et al.,*1987). One noteable feature of the *MTS1* protein is the predicted turns at the carboxy terminus of the protein. Also the carboxy terminus and the amino terminus are extremely hydrophilic, with the central region of the protein being relatively neutral.

6.6.4 Database Searches

6.6.4.1 Proline region

The proline rich amino terminal region of MTS1 protein from residue 1 to 130 was used to search the Swissprot database for proteins which were similar. Many of the proteins identified in this way only showed identitity or similarity with the proline residues. However, circumsporozoite protein precursor (Lal *et al.,* 1987), hsv11 large tegument protein (McGeoch *et al.,* 1988), and mouse proline-rich precursor protein PRP3 (Ann and Carson, 1985) have a greater level of similarity due to the presence of glutamine residues which are also present in MTS1 protein. In these other proteins, the proline and glutamine residues are often arranged in repeats together with other residues, and are proposed to form a strong structural domain. MTS1 also contains a degenerate repeat of APQEX(X) (Figure 6.5).

6.6.4.2 Glycine-Rich Region.

The *MTS1* protein is rich in glycine residues near the carboxy terminus (Figure 6.5). Several other proteins with glycine-rich regions show some

Figure 6.6

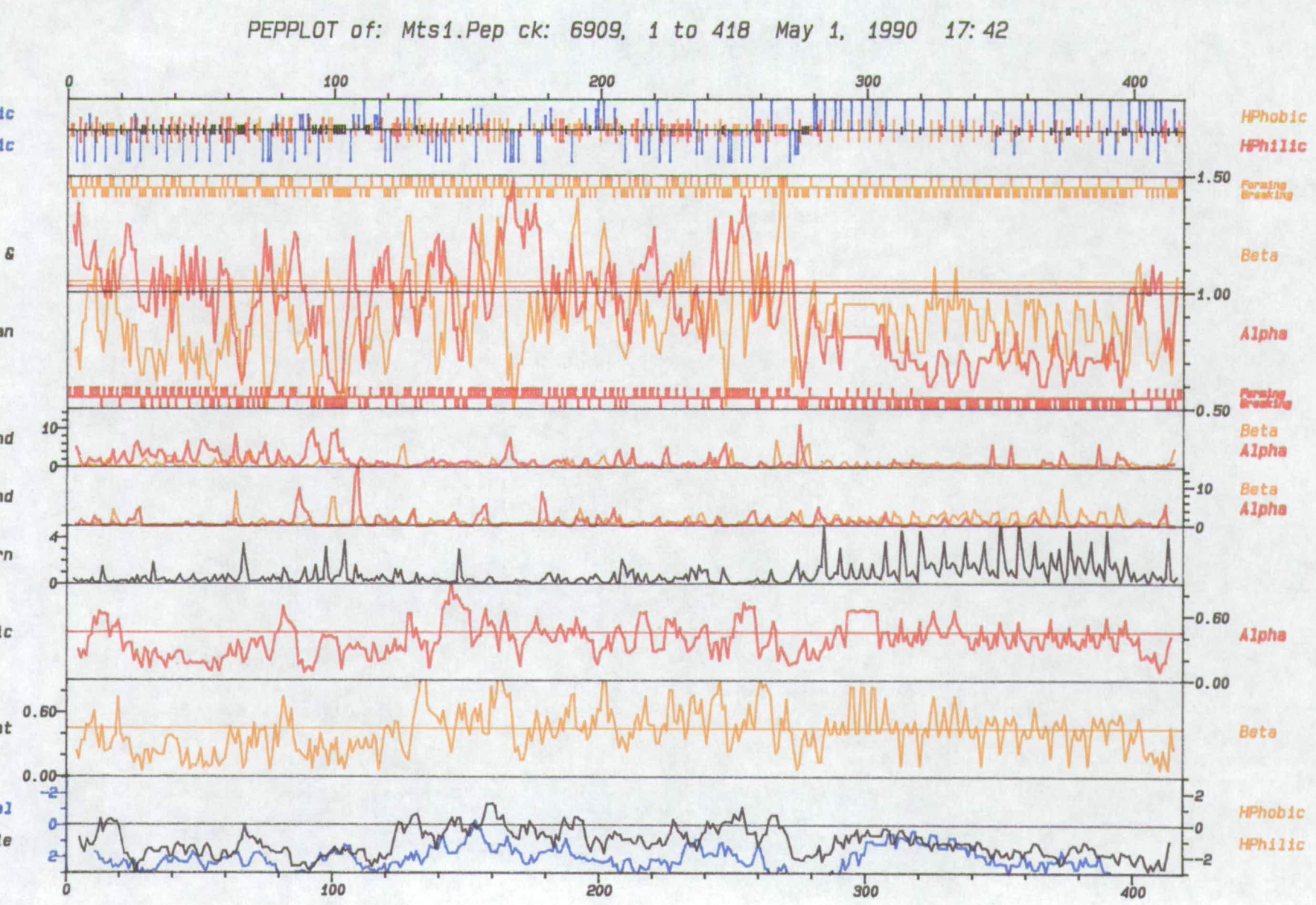

PEPPLOT of: Mts1.Pep ck: 6909, 1 to 418 May 1, 1990 17:42
0
100
200
300
400
Basic
Acidic
HPhobic
HPhilic
1.50
Forming
Breaking
Chou &
Fasman
Beta
Alpha
1.00
0.50
NH2 End
COOH End
Turn
10
4
Hydrophobic
0.60
0.00
Moment
Goldman et al
Kyte-Doolittle
-2
2

Figure 6.7

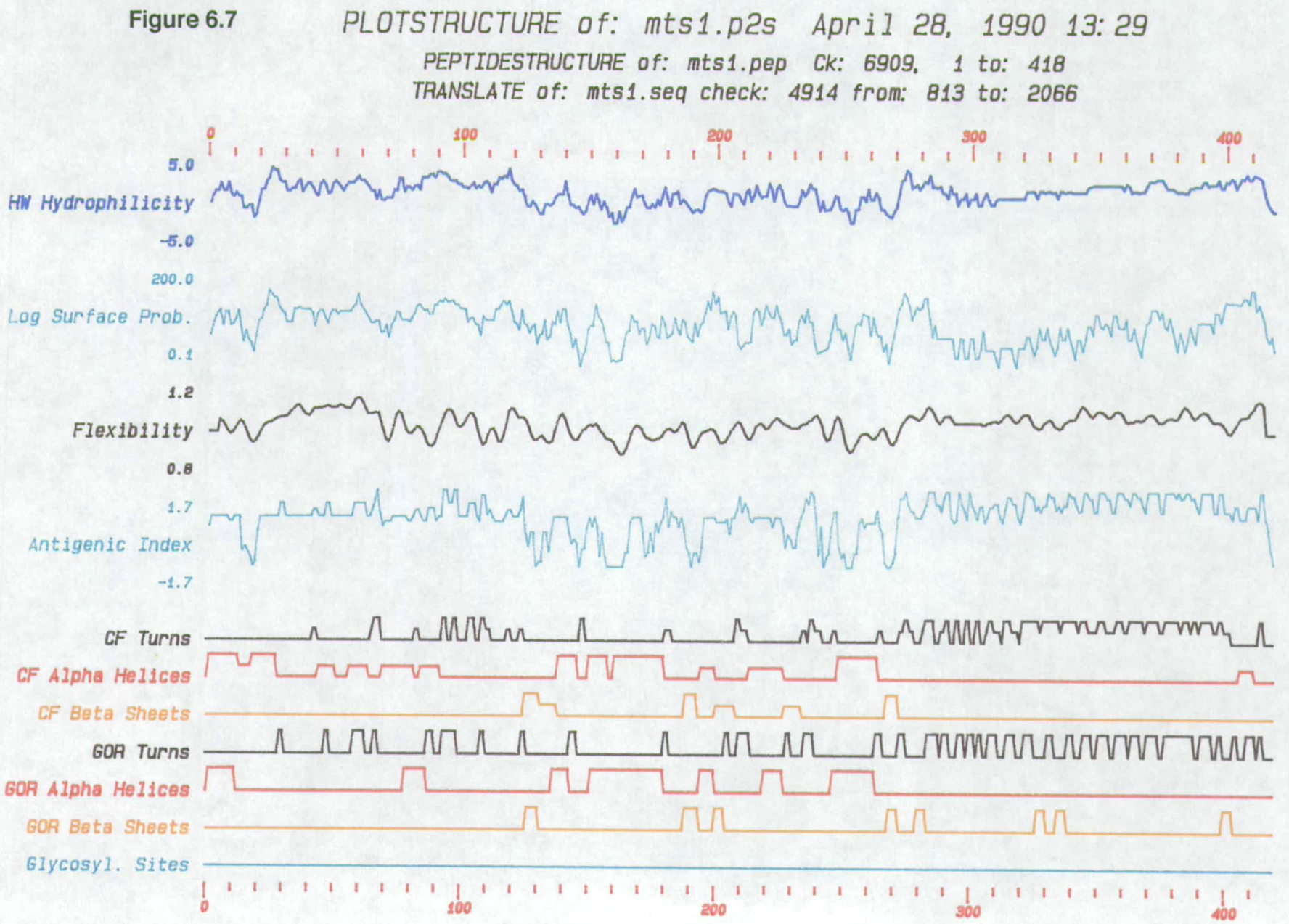
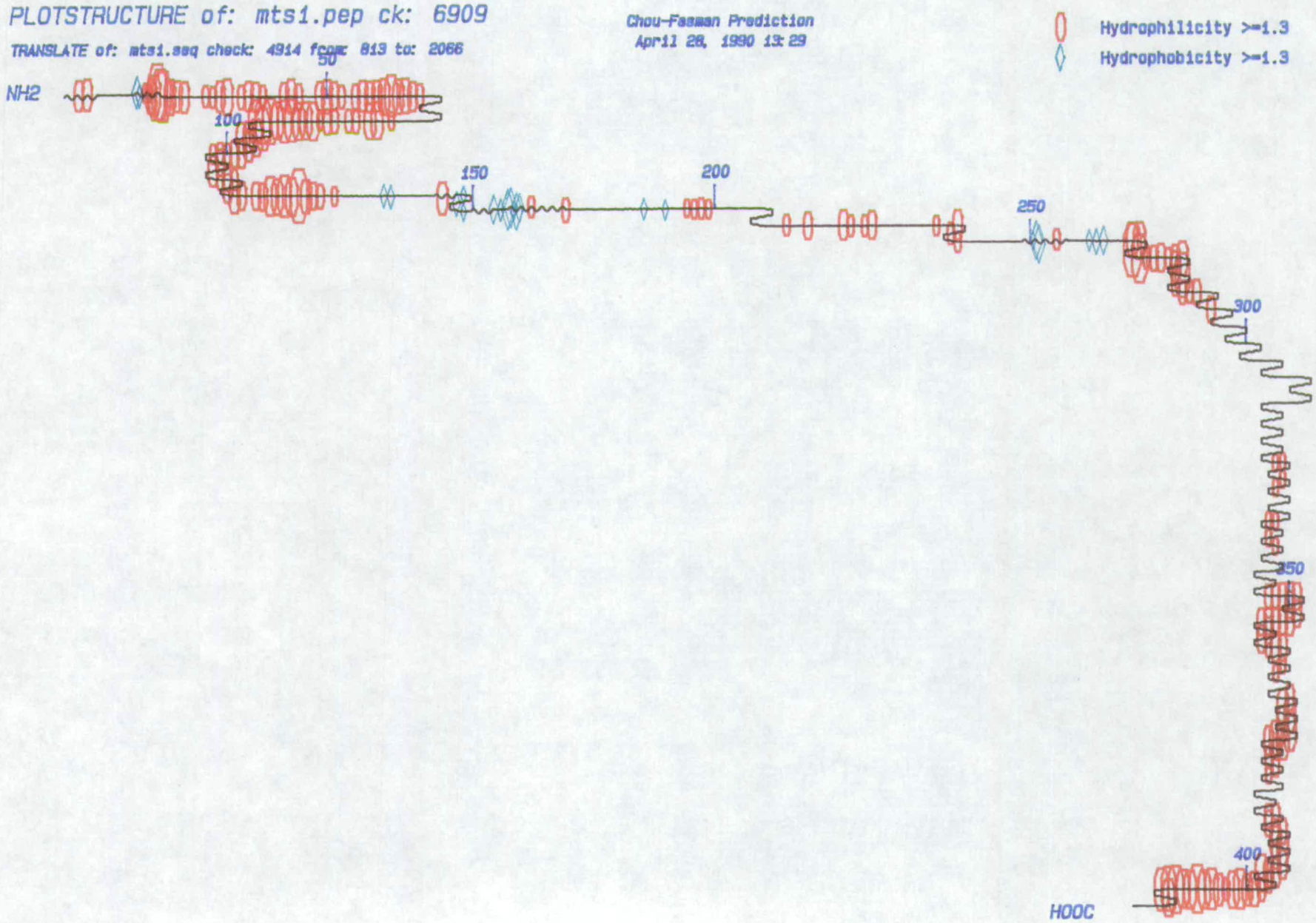

PLOTSTRUCTURE of: mts1.p2s April 28, 1990 13:29
PEPTIDESTRUCTURE of: mts1.pep Ck: 6909, 1 to: 418
TRANSLATE of: mts1.seq check: 4914 from: 813 to: 2066
0
100
200
300
400
5.0
HW Hydrophilicity
-5.0
200.0
Log Surface Prob.
0.1
1.2
Flexibility
0.8
1.7
Antigenic Index
-1.7
CF Turns
CF Alpha Helices
CF Beta Sheets
GOR Turns
GOR Alpha Helices
GOR Beta Sheets
Glycosyl. Sites

Figure 6.8


PLOTSTRUCTURE of: mts1.pep ck: 6909
TRANSLATE of: mts1.seq check: 4914 from: 813 to: 2066
Chou-Fasman Prediction
April 28, 1990 13:29
Hydrophilicity >=1.3
Hydrophobicity >=1.3
NH2
50
100
150
200
250
300
350
400
HOOC

similarity to the *MTS1* protein, and were identified as such from database searches using the residues of the glycine-rich region (residues 281-400). In some of these other proteins, the glycine-rich region has a structural role such as in cytoskeletal keratin (Klinge *et al.,* 1987); chorion eggshell proteins (Martinez-Gruzado *et al.,* 1988) and glycine-rich cell wall structural protein from kidney bean (Keller *et al.,* 1988). In these cases, the only feature in common with MTS1 is the high content of glycine residues. However, glycine-rich regions are also found in nucleic acid binding proteins such as nucleolin (C23) (Lapeyre *et al.,* 1987); single-stranded nucleic acid binding protein from yeast (SSB1) (Jong *et al.,* 1987); hnRNP A1 (Cobianchi *et al.,* 1986); abscissic acid inducible protein (Mortenson *et al.,* 1989); VASA protein from *Drosophila melanogaster* (Lasko and Ashburner, 1988); DED1 protein from *Saccharomyces cerevisiae* (Struhl, 1985) and CC63 protein from *Saccharomyces cerevisiae* (Hanic-Joyce *et al.*, 1987). In all these cases, the glycine residues are interspersed with arginine and phenylalanine residues, as is also found in MTS1, indicating that MTS1 may be related to this group of proteins.

Within the glycine rich region in the C-terminus of MTS1 are two slightly different separate motifs of RGGFRG and RGGYGG, both of which are tandemly repeated (Figure 6.5). A search for other proteins in the database with these motifs revealed that the RGGYGG motif occurs singly in chorion proteins (Martinez-Gruzado *et al.,* 1988), and glycine-rich cell wall structural protein (Keller *et al.,*1988). However, the RGGFRG pattern only occurs in nucleic acid binding proteins. The glycine-rich region of nucleolin contains only one copy of the RGGFRG motif, whereas the SSB1 protein (single-stranded nucleic acid binding protein) contains several repeats of this motif (Jong *et al.,* 1987), and the Epstein-Barr virus nuclear antigen polypeptide possesses one copy of RGGYGG (Rawlins *et al.,* 1985). The region of similarity between SSB1 and MTS1 is shown in Figure 6.9. It is not known what function these glycine-rich regions might have but it has been

Figure 6.9 Similarity Between MTS1 and SSB1

```
SSB1  129 GRGGFRGRGGFRGGFRGGYRGGFRGRGNFRGRAA 163
          .||||||||||||||||||:|||| :||.| |. :
MTS1  282 NRGGFRGRGGFRGGFRGGFRGGF.SRGGFGGPRG 315
```

Best fit alignment of the glycine-rich region of MTS1 to SSB1 (Jong et al., 1987). The repeating motif in MTS1 extends beyond that of SSB1.

Key

| - identical residues

: - very conservative substitutions

. - conservative substitutions

demonstrated that nucleolin does not require its glycine-rich C-terminus for binding to RNA (Bugler *et al.*,1987).

6.6.4.3 RNP consensus

As the MTS1 protein appears to to show some similarity to nucleic acid binding proteins, the amino acid sequence was visually examined for the presence of an RNP consensus sequence found in eukaryotic RNA binding proteins (Sachs *et al.*, 1986). The RNP consensus has a high concentration of positive charge followed by several closely spaced aromatic residues, and usually contains the motif GFAFV or GFGFV. It has been found in messenger RNA-binding proteins, preribosomal RNA-binding proteins, heterogeneous nuclear RNA-binding proteins and small nuclear RNA-binding proteins. One possible sequence was located between residues 158 and 166. The central region of the MTS1 protein covering the proposed RNP consensus (residues 121 to 270) was used to search the Swissprot protein database, and the most siginicant matches were found with proteins which are thought to bind RNA. These proteins such as poly(A)binding protein from human (Grange *et al.*, 1987) and yeast (Sachs *et al.*, 1986), hnRNP A1 protein from *Drosophila melanogaster* (Haynes *et al.*, 1987), hnRNP C (Swanson *et al.*, 1987), and nucleolin (Lapeyre *et al.*, 1987) are known to contain the RNP consensus. The comparisons shown in Figure 6.10 reveal that the RNP consensus in MTS1 shows a high degree of similarity to other RNP consensus sequences. The RNP consensus in nucleolin has been directly implicated in RNA binding (Bugler *et al.*,1987) and the presence of this sequence in MTS1 strongly suggests that it is an RNA binding protein.

6.7 DISCUSSION.

The cloning of the wild-type *MTS1* gene described in this Chapter has allowed several features of its gene product to be examined. Because of the cloning strategy adopted, the cloned MTS1 gene has been demonstrated to complement the temperature sensitive phenotype of *mts1* only. However, as this phenotype is genetically linked to the ability to suppress the defective

Figure 6.10 Sequence Homology Between MTS1 and other RNA binding proteins.

```
MTS1 Protein                          --VKIL--------NGFAFVEFEE--A----
hnRNP A1 (Drosophila)[1]              --GKK---------RGFAFVEFDDYDP----
Poly(A)-binding protein (Yeast)[2]    LSSKIATDE-NGKSKGFGFVHFEEEGAA-K-
Poly(A)-binding protein (Human)[3]    LSVKVMTDE-SGKSKGFGFVSFERHEDAQK-
Nucleolin (Hamster)[4]                ---RIVTDRETGSSKGFGFVDFNSEEDA-K-
AbaI (Maize)[5]                       ----------TGRSRGFGFVTFSSENSMLD-
SSB1 (Yeast)[6]                       --GRIFTSD-SAN-RGMAFVTFSGE------
hnRNP C (Human)[7]                    Y-GKIVG---CSVHKGFAFVQYVN-------
                                                 .**.**
```

Sequences were aligned using CLUSTAL.

KEY

* - match across all sequences

. - conservative substitutions

1. Haynes *et al.*, 1986.
2. Sachs *et al.*, 1986.
3. Grange *et al.*, 1987.
4. Lapeyre *et al.*, 1987.
5. Mortenson and Dreyfuss, 1989.
6. Jong *et al.*, 1987.
7. Swanson *et al.*, 1987

mitochondrial targeting signal (Section 5.3.2) there is a strong likelihood that the *MTS1* gene product is involved in the recognition of mitochondrial precursor proteins. The possible role of *MTS1* in the targeting pathway is discussed in the following chapter.

Southern analysis has revealed that the *MTS1* gene is a single gene, and this probably means that the other complementing clone obtained (pEE24) contains the same region of chromosomal DNA. Less stringent washings of the Southern blot should reveal whether there are any other genes with significant homology to *MTS1.* The *MTS1* gene appears to be essential for growth, and Northern analysis shows that it is transcribed as a 1.3 kb transcript.

Sequencing of *MTS1* has enabled comparisons to be carried out with other genes in nucleic acid and protein databases. From these comparisons, it is clear that the *MTS1* gene is a novel gene, and that it may encode an RNA binding protein of 48 kDa. The evidence for this relies on two regions within the predicted open reading frame: the glycine-rich region which is found in many RNA binding proteins, but the function of which is unknown; and the RNP consensus which is known to bind RNA (Bugler *et al.,* 1987). Many RNA-binding proteins also possess proline-rich regions, and this is further evidence that *MTS1* is an RNA-binding protein. The possible significance of this in understanding the role of the MTS1 protein targeting is described in Chapter 7.

CHAPTER SEVEN

DISCUSSION

7.1 Identification of Targeting Components.

Many previous studies have highlighted the importance of presequences in mitochondrial targeting process, but until recently few had concentrated on the components which interact with presequences. The identification of these components would allow several key questions to be answered. For example, are there chaperones which stabilize mitochondrial precursors? Can known chaperones such as trigger factor or GroEL substitute *in vitro,* or can their homologues be found *in vivo* ? Are there recognition factors which bind specifically to the precursor protein? Can receptors be isolated which can be crosslinked to the precursor? Essentially, the questions which remain to be answered would put mitochondrial targeting in perspective with other targeting systems - how much similarity is there between mitochondrial targeting and protein secretion, and what is the evolutionary significance of any similarity ?

Targeting components could be identified biochemically or genetically. In order to identify them biochemically, it would be useful to have sufficient precursor protein to perform binding experiments. The results in Chapter 3 provide evidence that the purification of a native precursor protein would only be feasible if a suitable expression system were found. As the precursor of β-subunit is relatively stable (Reid and Schatz, 1982a), it is a potential candidate for purification. However, expression of the *ATP2* gene in *E. coli* and *S. cerevisiae* is not as efficient as the expression of other proteins using identical systems (Dobson *et al.,* 1982). For this reason, it may be better to use another mitochondrial precursor protein which can be expressed at high levels. However, one potential problem is that the expression of any mitochondrial precursor in yeast may be detrimental to cell growth, and therefore it may be best to express precursor proteins in *E. coli.* Given that purification of a native precursor protein were achieved, then much could be learned about its interaction with other proteins in a similar way that the

purified bacterial precursor protein pro-OmpA has been used with *in vitro* systems (Lecker *et al.*, 1989).

As a biochemical approach was less fruitful than was initially hoped, a genetic approach is described in Chapter 5 of this thesis. Having defined a mutation in the presequence of β-subunit which gives a reduced interaction at an early stage in the targeting pathway (that is at the initial binding step; Chapter 4), extragenic suppressors were isolated. These should hopefully encode components of the targeting pathway which interacted with the precursor polypeptide, and may act by increasing the interaction of the precursor with an import component. A temperature sensitive nuclear mutation (*mts1*) was isolated.

7.2 The Role of *MTS1* in Mitochondrial Targeting.

The cloning of the *MTS1* gene by complementation of the temperature sensitive phenotype as described in Chapter 6 reveals that it encodes a protein of approximately 48 kDa. The MTS1 protein has a high abundance of proline and acidic residues at its amino terminus and a glycine-rich hydrophilic region at its carboxy terminus. Such a hydrophilic region would perhaps be capable of interacting with other proteins. The MTS1 protein also possesses an RNP consensus sequence which strongly suggests that it is an RNA binding protein. It appears then that the MTS1 protein may be part of a ribonucleoprotein complex. The genetic evidence of the involvement of the MTS1 protein in targeting suggests that suppression by the MTS1 protein is specific for the original targeting defect, and this strongly suggests that it is involved in an interaction with the precursor protein. It is still possible however that the MTS1 protein is not involved in targeting, and it affects the ability of cells to grow on glycerol by some indirect mechanism.

How might an RNA binding protein be involved in mitochondrial targeting? Comparison to other targeting processes demonstrates the involvement of ribonucleoprotein complexes in the recognition and targeting of precursor proteins. For example, Signal Recognition Particle from higher

eukaryotes is known to contain both RNA and protein components. Six different polypeptides of 9 kDa (SRP9), 14 kDa (SRP14), 19 kDa (SRP19), 54 kDa (SRP54), 68 kDa (SRP68) and 72 kDa (SRP72) and a 7S RNA have been identified. The RNA species is known to be required for the arrest of elongation of the precursor polypeptide, together with SRP9 and SRP14 proteins. SRP19 is involved in the binding of SRP54 to the 7S RNA. SRP19 has been cloned and sequenced and has been shown to bind to the 7S RNA *in vitro* (Lingelbach *et al.,* 1983). SRP68 and SRP72 are thought to be involved in the binding of SRP to the ER membrane.

Recently, an *E. coli* 4.5S RNA has been shown to be similar to SRP 7S RNA (Poritz, *et al.,*1988), and an *E. coli* protein of 48 kDa of unknown function (P48; Bystrom *et al.,*1983) shows extensive similarity to SRP54 (Romisch *et al.,*1989). It has been demonstrated that SRP 7S RNA can functionally replace *E. coli* 4.5S RNA *in vitro,* and also that SRP54 and *E. coli* P48 can both bind 4.5S RNA *in vitro* and *in vivo* (Romisch *et al.,* 1990). This indicates that *E. coli* possesses a ribonucleoprotein complex containing P48 and 4.5S RNA which is a homologue of SRP. A homologue of the 7S RNA has been identified in the fission yeast *Schizosaccharomyces pombe*, and is anticipated to occur in *Saccharomyces cerevisiae.*

The processes described above are involved in the export or secretion of proteins, and it is possible that they may be evolutionarily related. As there is some degree of functional similarity between secretion/export and mitochondrial protein targeting, it is possible that there is some similarity in terms of the components that are involved. That is, it is possible that the mitochondrial targeting process could have evolved from a primitive protein export pathway. For example, Hsp70 proteins are known to be required for both secretion and mitochondrial protein targeting in yeast. In this regard mitochondrial protein targeting has been shown to require an RNA species for efficient import *in vitro* (Firgaira *et al., 1984*). It is therefore a possibility that a ribonucleoprotein complex is involved in this process, and that the MTS1 protein is part of this complex. However, MTS1 does not show any similarity

to SRP54 or SRP19, or P48. None of these proteins possess the RNP consensus sequence, which suggests differences from MTS1 in their binding to RNA.

At what stages might the MTS1 protein be involved in the targeting process? As the *MTS1* gene was isolated as a suppressor of a known defect in a targeting sequence, it is possible that it interacts with the presequence directly. For example it may be a cytosolic recognition factor or a molecular chaperone. It has not been possible to establish the functional location of protein, but it cannot be ruled out that it is mitochondrially-associated and may be a membrane-bound receptor or translocase.

7.3 Future Studies

The cloning of the *MTS1* gene means that it may be possible to express the protein it encodes in *E. coli*. It will be possible to construct fusion proteins (such as β-gal::*MTS1* in *E. coli*). Purification of such fusion proteins and the subsequent raising of antisera against these proteins will allow the further biochemical characterization of the MTS1 protein.

Firstly it should be possible to implicate the MTS1 protein in the import process by adding antisera to an *in vitro* import assay. An inhibition of import would confirm that MTS1 is directly involved. Secondly, it should be possible to identify the cellular location of the MTS1 protein, initially by carrying out subcellular fractionation experiments to determine whether it is mitochondrially-located or cytoplasmically-located. More specifically, immunofluorescence studies should confirm its intracellular location. Thirdly, it should be possible to identify the RNA species which binds to the RNP consensus sequence in the MTS1 protein. This can be achieved by immunoprecipitation of the MTS1 protein from cell extracts, then protease digesting the immunoprecipitates and 3' end-labelling the RNA species with pCp and RNA ligase. Finally, antisera could assist in the purification of the MTS1 protein with the ultimate aim of reconstitution of the targeting machinery.

From the genetic point of view, it still has not been established how the temperature-sensitivity of the MTS1 protein has an effect on the growth of the cell. It was originally assumed that it was due to an inefficient interaction at the higher temperature with precursors which are essential for growth. However it is also possible that the MTS1 protein interacts with other proteins such as receptors, or may be part of a complex. In this respect, the temperature sensitivity may be due to an inefficient interaction with another component. It would therefore be feasible to isolate extragenic suppressors of the temperature-sensitive defect in the hope that they resided in other components, and would therefore allow their cloning and characterization.

In terms of targeting processes in general, the identification of essential targeting components involved will be vital to our understanding of the processes involved. Significant progress has been made towards reconstitution of the secretory pathway of higher eukaryotes, following the purification of SRP. It remains to be established what similarities and differences exist between the various targeting processes, and the reconstitution of mitochondrial targeting *in vitro* would greatly facilitate the achievement of this aim.

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